

Bacteriophage infection drives loss of β -lactam resistance in methicillin-resistant *Staphylococcus aureus*

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eLife Assessment

The manuscript explores how bacterial evolution in the presence of lytic phages modulates β -lactams resistance and virulence properties in methicillin-resistant *Staphylococcus aureus* (MRSA). This **important** work improves our knowledge of how mutation in genes required for phage infection confers sensitivity to β -lactams and alter virulence properties. Altogether, the findings are **convincing**.

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Abstract

Bacteriophage (phage) therapy is a promising means to combat drug-resistant bacterial pathogens. Infection by phage can select for mutations in bacterial populations that confer resistance against phage infection. However, resistance against phage can yield evolutionary trade-offs of biomedical relevance. Here we report the discovery that infection by certain staphylococcal phages sensitizes different strains of methicillin-resistant *Staphylococcus aureus* (MRSA) to β -lactams, a class of antibiotics against which MRSA is typically resistant. MRSA cells that survive infection by these phages display significant reductions in minimal inhibitory concentration against different β -lactams compared to uninfected bacteria. Transcriptomic profiling reveals that these evolved MRSA strains possess highly modulated transcriptional profiles, where numerous genes involved in *S. aureus* virulence were downregulated. Phage-treated MRSA exhibited attenuated virulence phenotypes in the form of reduced hemolysis and clumping. Despite sharing similar phenotypes, whole-sequencing analysis revealed that the different MRSA strains evolved unique genetic profiles during infection. These results suggest complex evolutionary trajectories in MRSA during phage predation and open up new possibilities to reduce drug resistance and virulence in MRSA infections.

Staphylococcus aureus is one of the most notorious and widespread bacterial pathogens, responsible for hundreds of thousands of severe infections worldwide every year. Methicillin-resistant *S. aureus* (MRSA) poses a particular clinical threat, as MRSA infections increase mortality, morbidity, and hospital stay, as compared to those caused by methicillin-sensitive *S. aureus* (MSSA).^{1,2} Part of MRSA's notoriety stems from its strong resistance against the β -lactam family of antibiotics, such as penicillins and cephalosporins, which inhibit the activity of transpeptidase

enzymes during peptidoglycan synthesis in bacterial cell walls.² β -lactams are one of the most commonly prescribed drug classes, with many designated as “Critically Important” antimicrobials by the World Health Organization.³ Thus, MRSA infections pose a considerable public health risk as they are notoriously difficult to treat and are widespread in communities and hospital settings. Indeed, in 2019, MRSA alone accounted for more than 100,000 deaths attributable to drug-resistant infections worldwide.⁴

A chief mediator of β -lactams resistance in MRSA is the SCCmec cassette, a mobile genetic element that carries the resistance gene *mecA*. *MecA* encodes for the penicillin-binding protein 2A (PBP2a), a transpeptidase that has a low affinity for β -lactams.⁵ This lower affinity permits PBP2a to participate in peptidoglycan synthesis even in the presence of β -lactams, ultimately resulting in cell survival. In addition to *mecA*, numerous MRSA strains also carry β -lactamases, such as BlaZ, that degrade β -lactams, thus further contributing to drug resistance. Together, these mechanisms can severely limit treatment options against MRSA, with current clinical treatment options relying primarily on vancomycin and daptomycin.⁶ Both vancomycin and daptomycin are last resort antibiotics against MRSA, and a major concern is the increasing resistance of MRSA to these drugs.^{7,8} Developing solutions to combat MRSA is a major focus in academia and industry.

Due to its drug resistance and clinical burden, *S. aureus* is a prime candidate for alternative antimicrobial treatments, such as bacteriophage (phage) therapy. Phages are viruses that infect and kill bacteria, posing one of the greatest existential threats to bacterial communities, with some estimates suggesting that 40% of all bacterial mortality worldwide is caused by phage predation.⁹ In phage therapy, lytic phages are administered to kill the bacterial pathogen(s) causing an infection. Phages offer certain advantages over traditional antibiotics: they are highly specific to their hosts by reducing off-target killing; they self-amplify and evolve, enabling the rapid generation of new phage variants with improved activities; and they are generally regarded as safe, as toxicity has been reported only in extremely rare cases in animals and patients.¹⁰ Indeed, against *S. aureus* infections, over a dozen promising case studies and clinical trials have been reported in the past decade.¹¹

Despite these advances, routine use of phage therapy is still met with challenges. Chief among these is the inevitable rise of phage resistance, as phage predation exerts a strong selective pressure on bacterial populations. According to one meta-analysis that focused on phage therapy outcomes, resistance against phage evolved in 75 percent of human clinical cases in which the evolution of resistance was monitored.¹² Mutations represent a chief pathway by which bacteria evolve resistance against phage. To date, the best characterized phage resistance mutations involve alterations on cell surface receptor molecules that mediate phage attachment. In many bacteria, these receptors are often proteins or sugar moieties, which are recognized by phage proteins.^{11,13,14} For example, in *Escherichia coli*, mutations in the cell-wall protein LamB confer resistance against *lambda* phage infection, while in *S. aureus*, mutations that modify wall teichoic acid (WTA) have been shown to limit phage infection.^{14,15} Complicating the picture, studies have revealed that a plethora of additional host mechanisms, including dedicated anti-phage defense systems, can impact the evolution of resistance against phage.^{16,17} These problems highlight the importance of developing phage treatment strategies that minimize or capitalize on the evolution of phage resistance.¹⁸

A unique aspect of phage therapy is the possibility to exploit evolutionary trade-offs to combat resistant pathogens. A genetic trade-off is defined as an evolved trait that confers a fitness advantage against a particular selective pressure at the expense of reduced fitness against an unselected pressure. Across many different species of bacteria, such trade-offs have been shown to occur between phage resistance and antibiotic resistance (**Figure 1A**). Phages that bind to a virulence factor or mechanism for antibiotic resistance in the target bacteria are predicted to exert a strong selection pressure on the bacteria to mutate or downregulate the phage-binding target. These changes would confer protection against phage infection but could in turn reduce the

resistance or virulence in the bacterium. As an example, in *P. aeruginosa*, infection by the phage OMK01, which binds to the outer membrane protein M OprM of MexAB- and MexXY-OprM efflux pumps, drives the evolution of mutations in those genes, leading to the re-sensitization of phage-resistant *P. aeruginosa* mutants to antibiotics.^{10,19}

Little is known about how phage resistance can mediate genetic trade-offs in MRSA. Previous work has shown that phage resistance in *S. aureus* can proceed through genetic mutations that are not directly involved in phage binding. For example, studies by Berryhill and colleagues demonstrated that phage infection of *S. aureus* Newman, an MSSA strain, can select for mutations in *femA*, which is a cytoplasmic enzyme that catalyzes the formation of the pentaglycine bridge of peptidoglycans in *S. aureus*.²⁰ Rather than serving as a direct phage receptor molecule, *femA* maintains the integrity of the cell wall, which in turn could be vital for WTA maturation and phage attachment.^{13,21} Interestingly, a consequence of these *femA* mutations is increased sensitivity against antibiotics. We therefore asked how phage resistance might impact the physiology of drug-resistant MRSA, with the hopes of identifying genetic trade-offs of potential biomedical relevance.

In the work reported here, we show that infection by staphylococcal phages causes MRSA strains to evolve sensitivity to different types of β -lactam antibiotics and attenuate virulence phenotypes. We found that this loss of resistance and virulence is associated with distinct mutational profiles distinct in each MRSA strain, and that phage-treated, evolved MRSA populations display significant transcriptome remodeling. Unexpectedly, we also discovered a mutant phage with higher activity and a broader host range against MRSA. Findings from our work can help in the development of phage therapies that reduce drug resistance and virulence in pathogenic bacteria.

Results

Identification of Φ Staph1N with activity against multiple MRSA strains

For our studies, we focused on three MRSA strains – MRSA252 (USA200), MW2 (USA400), and LAC (USA300). All three MRSA strains are pathogenic isolates implicated in human disease and are used as representative examples for studying MRSA.^{22–24} To test for phage susceptibility, we performed plaquing assays with a panel of staphylococcal phages (**Table S1**, **Figure S1**). Of the phages tested, one phage, Φ Staph1N, which belongs to the *Kayvirus* genus, formed plaques on all three MRSA strains (**Figure 1B**).^{21,25} Yet, despite its ability to infect all three strains, Φ Staph1N infection was unable to eradicate MRSA cultures. Both MW2 and LAC displayed incomplete lysis in liquid culture at multiplicities of infection (MOIs) of 0.1 or lower (**Figure S2**). Furthermore, infected cultures of all three MRSA strains could recover back to high cell density after passaging one percent of the culture into fresh media following 24 hours of initial infection. These results suggest that infection by Φ Staph1N selects for resistant mutants that could sweep the population. Indeed, Φ Staph1N was unable to form plaques on recovered MRSA cultures that survived in the initial Φ Staph1N infection (**Figure 1B**).

Resistance against Φ Staph1N infection sensitizes MRSA against β -lactams

Because both phages and β -lactams interface with the bacterial cell wall, we hypothesized that resistance against Φ Staph1N infection could cause a trade-off in β -lactam resistance in MRSA even in the presence of PBP2a and BlaZ. We first tested the β -lactam sensitivity of the parental MRSA252, MW2, and LAC strains. As expected, all three strains displayed high minimal inhibitory concentrations (MICs) of ≥ 48 μ g/mL against the β -lactams oxacillin (OXA), cefazolin (CEF), amoxicillin (AMX), and amoxicillin & clavulanic acid (AMX+CA), visually indicated by their ability to form lawns surrounding antibiotic strips (**Figure 1C**, **Table S2**). The strains were sensitive

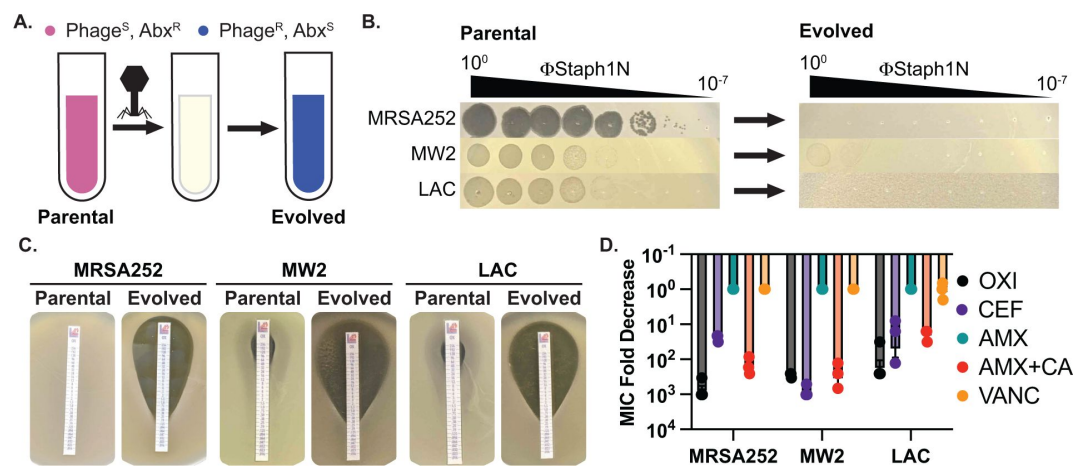


Figure 1.

Infection by bacteriophage $\Phi\text{Staph1N}$ drives the loss of β -lactam resistance in MRSA.

A. Schematic of the experimental setup. Drug-resistant (Abx^R), phage-sensitive (Phage^S) bacterial cultures are infected with phage. The population of infected cells is passaged and allowed to recover. The surviving cell population is resistant to phage infection (Phage^R) but has evolved sensitivity to antibiotics (Abx^S). **B.** $\Phi\text{Staph1N}$ infects MRSA strains MRSA252, MW2, and LAC (left panel). Following infection with $\Phi\text{Staph1N}$, evolved cultures of the three MRSA strains are resistant to $\Phi\text{Staph1N}$ (right panel). **C.** $\Phi\text{Staph1N}$ -treated, evolved MRSA strains show significant loss of resistance against oxacillin, compared to the parental strains. Loss of resistance is indicated by the area of bacterial clearance surrounding the antibiotic resistance strip. **D.** $\Phi\text{Staph1N}$ treatment causes loss resistance against different β -lactams. Plotted are the fold reductions of minimal inhibitory concentration (MIC) between treated and mock-treated cells. OXI = oxacillin; CEF = cefazolin; AMX = amoxicillin; AMX+CA = amoxicillin & clavulanic acid; VANC = vancomycin.

to vancomycin (VANC; MICs = 1.5 µg/mL), which inhibits cell wall synthesis through a different mechanism than β-lactams. Strikingly, phage-resistant MRSA that survived ΦStaph1N infection displayed a strong reduction in resistance against OXA, CEF, and AMX+CA, with fold reductions in MIC between 10 and 1000-fold (**Figure 1D**); no change in MIC was observed with VANC or with AMX alone. These results show at a phenotypic level that ΦStaph1N-resistant MRSA loses resistance towards most β-lactams.

We next asked whether this loss of β-lactam resistance depended on the MOI of ΦStaph1N. We infected the three MRSA strains with ΦStaph1N at MOIs ranging from 10^{-2} to 10^{-5} , isolated the surviving MRSA cells, and tested their MIC against oxacillin (**Table S2**). For MRSA252, we still observed a ~3-order of magnitude fold reduction of MIC at an MOI of 10^{-5} . With MW2, the reduction of MIC was markedly decreased with lower phage levels, showing no significant loss at MOIs of 10^{-3} or lower. For LAC, two replicates displayed a reduction of MIC by an order of magnitude at an MOI of 10^{-4} , while the third replicate did not display any change. These results show that for MRSA252, ΦStaph1N MOIs as low as 10^{-5} can still drive the loss of resistance, while for MW2 and LAC, higher MOIs of phage are needed to ensure the same outcome of reduced β-lactam resistance.

Discovery of a mutant ΦStaph1N with enhanced activity against MRSA

For ΦStaph1N, we noticed that while the phage could plaque on all three MRSA strains, its plaque-forming efficiency was reduced on the MW2 and LAC strains (**Figure 1B**, **Figure S1**). ΦStaph1N plaques on MW2 and LAC bacterial lawns were hazy, and the overall efficiency of plaquing was approximately two orders of magnitude less than that on MRSA252. Unexpectedly, we consistently observed smaller, clear plaques arising in the larger, hazy plaques of LAC (**Figure S3A**); notably this did not appear in MW2. We hypothesized that these clear plaques were caused by a mutant form of ΦStaph1N that evolved higher lytic activity. We isolated phage clones from these single plaques and tested their activity against MRSA. This mutant phage, which we called Evo2, plaques on LAC and MW2 strains with higher efficiency, displaying comparable plaquing to MRSA252 (**Figure 2A**). In growth experiments, we further observed that Evo2 lyses MRSA cultures at lower MOIs compared to ΦStaph1N (**Figure S2**). Evo2 exhibits lytic activity against MW2 and LAC even at an MOI of 10^{-4} , a concentration at which ΦStaph1N does not show any detectable activity against the two strains.

We sequenced the genome of Evo2 to determine the genetic mechanism driving this enhanced activity. We observed a single point mutation in ORF141 that induces a premature stop codon (**Figure S3B**). Sequence analysis with HHpred predicts ORF141 to be a putative DNA binding protein with an HTH motif (PDB: 2LVS, E-value: 2.5×10^{-9}). We speculate that this protein is a transcriptional regulator that when inactivated by a nonsense mutation, increases ΦStaph1N infectivity. Future studies will center on determining the mechanism of this mutation and why Evo2 only evolved in the LAC strain.

Given Evo2's enhanced activity against MRSA, we asked how predation by Evo2 affected β-lactam resistance. We infected MRSA252, MW2, and LAC with Evo2 at an MOI of 0.1 and measured the MICs against β-lactams after 48 hours of passaging. Similar to ΦStaph1N, infection by Evo2 reduced the MICs of the three MRSA strains against OXA, CEF, and AMX+CA, while MICs against AMX alone and VAN did not change significantly (**Figure 2B**). Expanding on the different classes of antibiotics, we tested whether Evo2 predation could impact the susceptibility to the transcription inhibitor rifampicin; the translation inhibitors erythromycin and mupirocin; and the cell envelope disruptors teicoplanin, fosfomycin, and daptomycin (**Table S3**). We found that the MICs of these antibiotics did not change significantly, with a few exceptions: in some cases, Evo2-

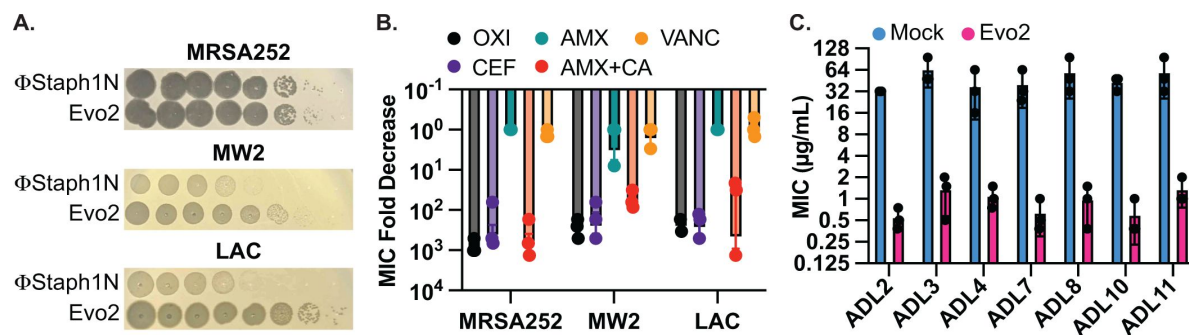


Figure 2.

Evo2 is a variant of ΦStaph1N with higher activity against MRSA.

A. Evo2 shows comparable infectivity towards MRSA252 but improved infectivity towards MW2 and LAC, relative to ΦStaph1N. The same plaquing data is also shown in [Figure S3A](#). **B.** Similar to ΦStaph1N, Evo2 infection reduces β-lactam resistance in MRSA. **C.** Evo2 infection reduces the MIC against oxacillin in clinical isolates of USA300 (ADLs).

resistant LAC became sensitized to fosfomycin and daptomycin; furthermore, one replicate of Evo2-resistant MRSA252 evolved sensitivity to teicoplanin. However, overall the MIC reduction in these cases was not as dramatic as the MIC reduction seen against β -lactams.

Finally, we examined how different MOIs of Evo2 impacted β -lactam resistance in MRSA (**Table S2**). We infected the three MRSA strains with Evo2 at varying MOIs from 10^{-2} to 10^{-5} and measured the MIC against oxacillin of the evolved MRSA. Across all three strains, we found that replicate cultures across different MOIs were unable to recover growth following Evo2 infection (**Table S2**). However, cultures of MRSA252, MW2 and LAC that did regrow displayed a loss of oxacillin resistance, between 10- to 1000-fold. Thus, overall Evo2 displayed a higher infectivity against the MRSA and a greater potency in reducing β -lactam resistance.

Evo2 is broadly active against recent clinical isolates of *S. aureus* USA300

MRSA252, MW2, and LAC were isolated in 1997, 1998, and in the early 2000s, respectively. We therefore tested if Evo2 can infect more recent *S. aureus* clinical isolates. We compared the plaquing efficiency of Evo2 and Φ Staph1N against 30 USA300 strains that were isolated between 2008 and 2011 at St. Louis Children's Hospital (**Table S1**).²⁶ We observe dramatic variation in the plaquing efficiency of Φ Staph1N and the 30 strains, while Evo2 exhibited a higher plaquing efficiency in the majority of the 30 strains (**Table S4**). We then tested how infection of Evo2 impacted OXA resistance in 12 (ADL1-12) of these clinical isolates. We infected the strains with Evo2 at an MOI of 0.1 and, if a phage-resistant population was recovered, measured the OXA MIC after 48 hours of passaging. Interestingly, after 15 independent challenges with Evo2, we were not able to recover phage-resistant populations from ADL1, 5, 6, and 12. This suggests that Evo2 resistance acquisition is a rare event in these strains. In the rest of the strains, we observed that similarly to MRSA252, MW2, and LAC, the OXA MIC was reduced between 10-to 100-fold after Evo2 infection (**Figure 2C**). Overall, these results highlight the broader host range and activity of Evo2.

Effect on β -lactam MIC by other phages

We asked whether two additional phages from our collection could elicit MIC reduction against oxacillin in MRSA. The MRSA LAC strain is sensitive to infection by Φ NM1 γ 6, a lytic version of the temperate phage Φ NM1 of the *Dubovvirus* genus, derived from the *S. aureus* Newman strain.²⁷ In LAC, Φ NM1 γ 6 displays plaquing comparable to that of Φ Staph1N, while also showing activity against some of the clinical USA300 isolates (**Figure S1**, **Table S4**). Therefore, we infected LAC with Φ NM1 γ 6 at an MOI of 0.1 and measured the MIC against β -lactams of the surviving cells. While the recovered LAC cultures exhibited resistance against Φ NM1 γ 6, they did not show a reduction in MIC (**Figure S4**), suggesting that phage resistance caused by Φ NM1 γ 6 is uncoupled from β -lactam resistance. We also isolated from the environment a second phage of the *Kayvirus* genus, called SATA8505,³⁰ and tested its activity against MRSA. SATA8505 is active against MRSA252, MW2, and LAC (**Figure S5**), and infection of the three strains caused a rise of phage resistance in MRSA252, MW2, and LAC; (**Figure S5A**). Similar to Φ Staph1N and Evo2, cells resistant to SATA8505 showed a strong loss of oxacillin resistance (**Figure S5B**). Altogether, our results with these two phages suggest that the ability to reduce β -lactam resistance is not a universal feature across all staphylococcal phages and call for a more comprehensive analysis of staphylococcal phages and their ability to elicit β -lactam trade-offs.

Genomic mutations in MRSA strains following phage infection

Following our phenotypic analyses, we examined the genomes of the phage-resistant MRSA. We first sequenced the genomes of three clonal isolates (A-C) from each MRSA strain that underwent Φ Staph1N, Evo2, or a mock infection. We observed that each MRSA strain evolved distinct mutation profiles (**Figure 3A**). Irrespective of the strain and phage treatment, most mutations

were predicted to be substitutions, followed by truncations (**Figure S6**). Cluster of Orthologous Genes (COG) variants associated with transcription, cell wall/membrane/envelope biogenesis, coenzyme transport and metabolism, and defense mechanisms were the most commonly found categories. Mutations in annotated genes that appeared at least twice across the clonal replicates are summarized in **Table 1**. Information on all detected genetic variants is listed in **Supplementary Table 1**.

One plausible hypothesis explaining the loss of β -lactam resistance is that phage infection selected for a defective *SCCmec* or *blaZ*. However, we did not observe any mutations in the two loci. Instead, all MRSA strains exhibited mutations in ancillary genes implicated on the loss in β -lactam resistance. In MRSA252, both Φ Staph1N and Evo2 infection were selected for frameshift or nonsense mutations in the *femA* gene that would inactivate the protein product. As discussed above, *femA* is required for the synthesis of the pentaglycine branch on *S. aureus* Lipid II, the peptidoglycan precursor (**Table 1**). Deletions of *femA* have been shown to increase susceptibility to β -lactams even when PBP2a (encoded by *mecA*) is expressed, thus providing a genetic mechanism for how some MRSA252 cells lose β -lactam resistance after phage selection.^{43,44} At the same time, we found the presence of other uncharacterized mutations in phage-resistant MRSA252. For example, clone A of Φ Staph1N-treated cells carried 2 mutations: a frameshift in *femA* and a substitution mutation in an uncharacterized protein; meanwhile, clone B displayed a substitution mutation in *pfkA*, a predicted ATP-dependent 6-phosphofructokinase and mutation in an intergenic region; clone C showed a substitution mutation in a putative transport protein, called *yueF* (**Figure 3B**, **Supplementary Table 1**). The role of these mutations in mediating phage resistance or β -lactam sensitivity, if any, remains unknown.

In MW2, we found mutations in two transcriptional regulators, *mgrA* and *sarA* (**Figure 3A**, **Table 1**). Both *mgrA* and *sarA* belong to the family of MarR (multiple antibiotic resistance regulator)/SarA (staphylococcal accessory regulator A) proteins, which regulate drug resistance and virulence in *S. aureus*.^{31–33} In Φ Staph1N-treated MW2, only *mgrA* was mutated, while in Evo2-treated MW2, clones also showed nonsense mutations in *sarA*. We also found mutations in *metK* and *ytqA* which are both predicted to be associated with S-adenosylmethione (SAM): *metK* synthesizes SAM, while *ytqA* belongs to the radical SAM enzyme family and is predicted to be involved in tRNA modification.^{41,47} Phage-treated MW2 also displayed mutations in other genes, including *tcaB* and *murE*. Notably, each clonal replicate had multiple mutations in the genome, while by contrast untreated MW2 cells only displayed a deletion in an intergenic region that is not present in any of the phage-treated samples. These findings suggest that MW2 could be amassing multiple mutations during the course of phage infection.

For LAC, we observed a third, distinct mutational pattern (**Figure 3A**). Of note, we found nonsense mutations in *arlR*, which is part of the *arlRS* two-component signaling system (**Table 1**). The activity of *arlRS* has been implicated in *S. aureus* virulence, pathogenicity, and oxacillin resistance.^{36,37} Further, we observed substitution mutations in *spoVG*, which is a transcription factor regulating the expression genes involved in a variety of functions, including cell wall metabolism (**Table 1**).³⁸ Indeed, *spoVG* activates the expression of *femA*.³⁹ Studies have shown that *spoVG* modulates β -lactam antibiotic resistance by modulating cell wall synthesis.³⁹ Similar to the other two MRSA strains, phage-treated clones of LAC showed multiple mutations in their genomes. We observed mutations in *prsA* and *bioA* that appeared in both the mock and phage treatment conditions, suggesting that these mutations do not arise due to phage selection.

Altogether, our results show that phage-infected MRSA strains acquire distinct mutational profiles. These mutations likely work in concert to promote phage resistance and β -lactam sensitivity, making it challenging to determine the mechanistic contributions of individual mutations. For example, we observed that the genes *mgrA*, *sarR*, and *arlR* evolved nonsense mutations, which would result in truncated, potentially non-functional protein products. We therefore tested if

Figure 3.

Phage infection of MRSA strains produces distinct mutational profiles.

A. Coding sequences (CDS) with mutations from the three MRSA strains following phage treatment or mock treatment. For each strain, three isolates were sequenced and their mutations identified. Mutations are color-coded based on the number of occurrences among the three replicates. **B.** Categories of genes with mutations that arose in each MRSA strain and treatment condition.

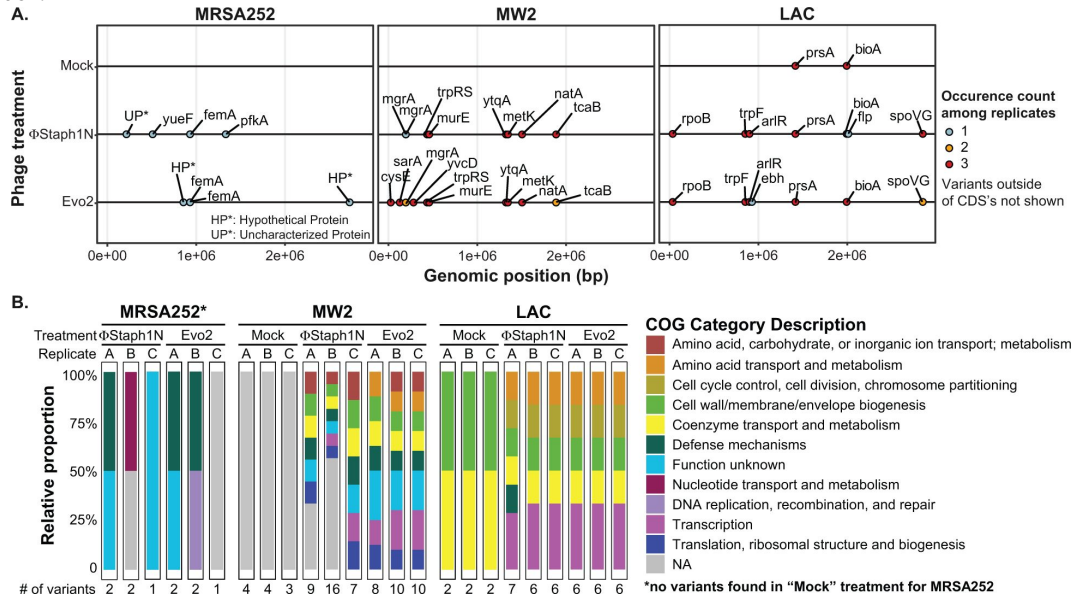


Table 1.

Mutated genes in MRSA following infection with phages ΦStaph1N or Evo2

Gene	Description	Strain	Phage infection	Reference
<i>sarA</i>	Transcriptional regulator of antibiotic resistance and virulence	MW2	Evo2	31,32
<i>mgrA</i>	Transcriptional regulator of antibiotic resistance and virulence	MW2	ΦStaph1N, Evo2	33,34
<i>rpoB</i>	Beta subunit of RNA polymerase	LAC	ΦStaph1N, Evo2	35
<i>arlR</i>	Transcriptional regulator of antibiotic resistance and virulence	LAC	ΦStaph1N, Evo2	34,36,37
<i>spoVG</i>	Transcriptional regulator of antibiotic resistance and virulence	LAC	ΦStaph1N, Evo2	38,39
<i>cysE</i>	Cysteine and methionine synthesis, serine O-acetyltransferase	MW2	Evo2	40
<i>metK</i>	Cysteine and methionine synthesis, S-adenosylmethionine (SAM) synthetase	MW2	ΦStaph1N, Evo2	41
<i>trpF</i>	Phenylalanine, tyrosine and tryptophan synthesis, phosphoribosylanthranilate isomerase	LAC	ΦStaph1N, Evo2	42
<i>femA</i>	Peptidoglycan synthesis, pentaglycine synthesis	MRSA252	ΦStaph1N, Evo2	43,44
<i>murE</i>	Peptidoglycan synthesis, UDP-MurNAc tripeptide synthesis	MW2	ΦStaph1N, Evo2	45
<i>trpS</i>	Aminoacyl-tRNA synthesis, tryptophanyl-tRNA synthesis	MW2	ΦStaph1N, Evo2	46
<i>ytqA</i>	tRNA modifications, mnm ⁵ s ² U synthesis	MW2	ΦStaph1N, Evo2	47
<i>yvcD</i>	Unknown	MW2	Evo2	
<i>natA</i>	ABC transporter	MW2	ΦStaph1N, Evo2	48
<i>tcaB</i>	Predicted multidrug efflux pump	MW2	ΦStaph1N, Evo2	49
<i>femC</i>	Fem-like factors	LAC	ΦNM1γ6	50

single knockout mutants of these genes alone are sufficient to confer resistance to Φ Staph1N and Evo2 (**Figure S7**). In MW2, the *mgrA* knockout resulted in a modest reduction in plaquing of Evo2 and Φ Staph1N. However, none of the remaining mutants in either the MW2 or LAC background conferred resistance. Prior experimental studies have also shown that phage resistance in *S. aureus* can arise from the disruption of single genes directly involved in the synthesis and modification of WTA, such as *tagO*.⁵¹ Some MRSA strains also alter cell wall glycosylation through dedicated genes encoded on prophages.¹⁴ However, we did not see any mutations in genes directly involved in WTA synthesis. Our results thus highlight how MRSA can take on unique mutational pathways under phage selection.

Finally, we examined the mutational profile in Φ NM1y6-resistant LAC populations. Because infection with Φ NM1y6 did not result in a decrease in OXA resistance, we hypothesized that mutations that arose in LAC following Φ NM1y6 would be distinct from those following Evo2 infection. We found that the two genes were mutated across three clonal isolates from different resistant populations: *bioA* and *fmhC*. As seen previously, mutations in *bioA* appeared in the mock treatment, suggesting that the mutations arose independently of phage selection. On the other hand, LAC showed a missense mutation in *fmhC* (H21D, **Table 1**, **Supplementary Table 1**). *FmhC* and its homologue *fmhA* pair with *femA* and *femB* to incorporate Gly-Ser dipeptides into peptidoglycan cross-bridges.⁵⁰ However, the mechanism of the H21D mutation is unknown, and to our knowledge, mutations in *fmhC* have not been associated with phages resistance in *S. aureus* before.

Phage-treated MRSA strains display broad changes to their transcriptomes

Our genomic analysis revealed that phage-treated MRSA evolved mutations in a variety of transcriptional regulators, some of which are known to affect MRSA virulence. We therefore hypothesized that the mutations in these regulators would fundamentally alter the transcriptional profile of the treated MRSA. To test this, we performed bulk RNA-seq experiments on MW2 and LAC strains that were treated with the phage Evo2 and compared their transcription profiles to those of untreated strains (**Figure 4**, **Table S5**). We observed significant changes in gene expression in both MW2 and LAC. Notably, mirroring the trend seen in the mutational data, we did not observe significant changes in the expression of genes in the *SCCmec* cassette or *blaZ* present in both MW2 and LAC.

We first compared our expression data against transcriptomic studies from previous studies. For example, Horswill and colleagues have shown that deletions of *arlRS* and *mgrA* de-represses the extracellular matrix binding protein *ebh*, resulting in significantly higher expression levels of the gene. In addition, these deletions also increased the expression of urease genes involved in the urea TCA cycle.^{33,37} In our experiments, both MW2 and LAC strains evolved nonsense mutations in *mgrA* and *arlR*, respectively. We therefore hypothesized that these mutations would mimic the effects of gene deletions and likewise yield elevated transcript levels of *ebh* and urease genes. Aligning with our hypothesis, differential expression data from both MW2 and LAC displayed a log₂ fold changes of >7 for *ebh* and >3 for *ureABCDEFG* genes (**Table S5**).

Additionally, both MW2 and LAC strongly upregulated several genes involved in cell wall maintenance. These include *lytN*, which is a murein hydrolase involved in the cross-wall compartment of *S. aureus*, and *fmhC*, which, as described previously, incorporate Gly-Ser dipeptides into pentaglycine cross-bridges in the *S. aureus* peptidoglycan cell wall. Overexpression of *lytN* has been shown to damage the cell wall, which in turn is alleviated by overexpression of *fmhC*.⁵⁰ Interestingly, *fmhC* overexpression is linked to increased β -lactam sensitivity and thus may contribute to the loss of β -lactam resistance phenotypes we observed in the MRSA strains.

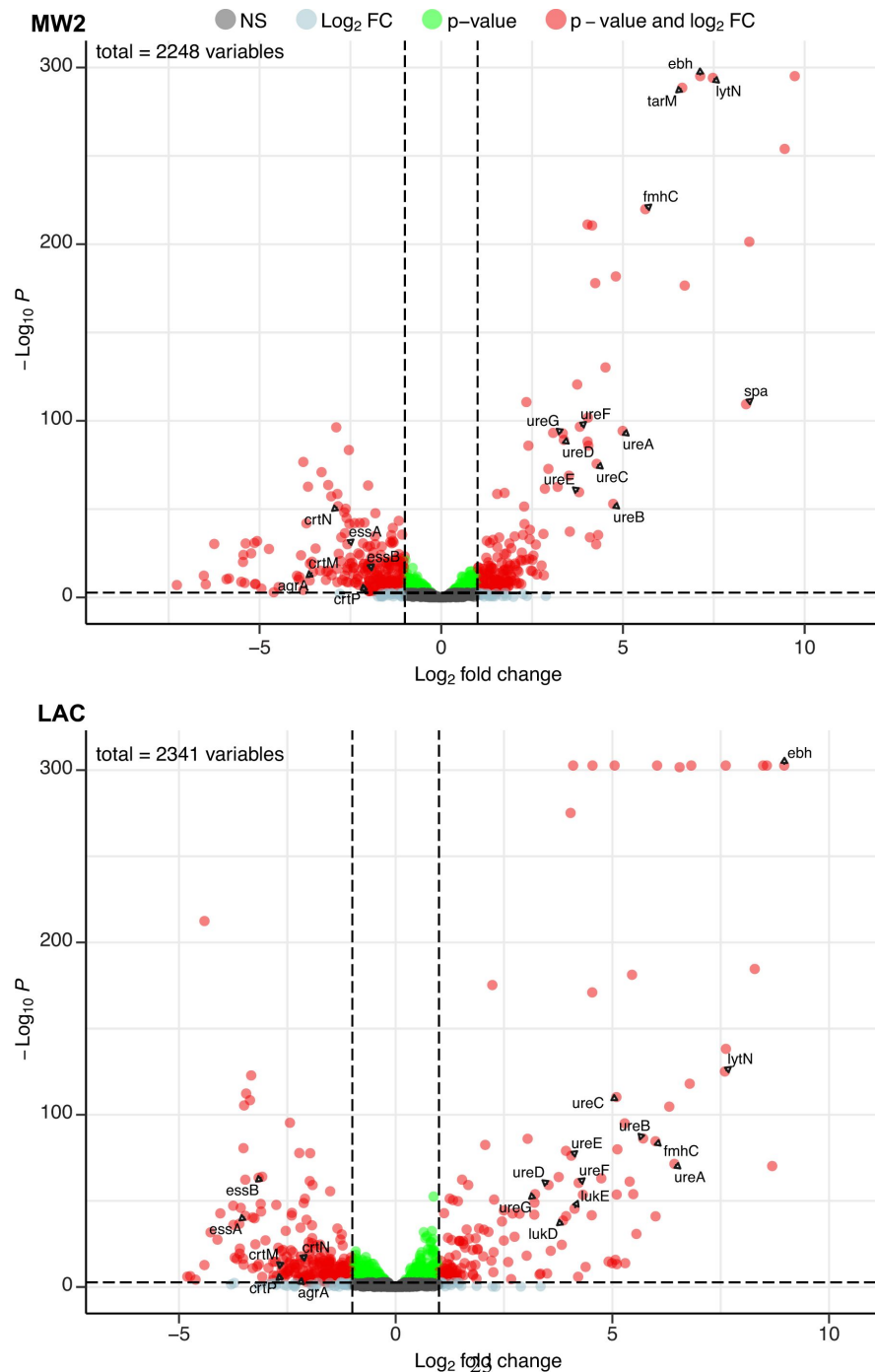


Figure 4.

Phage infection changes the transcriptomic profile of MRSA.

Differential expression analysis was performed on the transcriptomes of MW2 (top panel) and LAC (bottom panel). For both strains, Evo2-infected samples were compared to uninfected controls. 3 biological replicates were analyzed for each condition. Horizontal dotted lines represent an adjusted p-value cut-off of 0.002, while vertical dotted lines represent a log₂ fold change of -2 or 2 in expression. Transcripts with a log₂ fold change between -2 or 2 and a p-value >0.002 are labeled as grey dots (Not significant, NS); transcripts that pass either the fold change or p-value cutoff (but not the other) are represented as blue and green dots, respectively; transcripts that pass both cutoffs are shown as red dots. Genes discussed in the main text are labeled. Data for all the transcripts with significant fold changes is shown in [Table S5](#).

MW2 and LAC also downregulated numerous genes, many of which are known virulence factors. Both strains reduced transcript levels of genes in the locus of the type VII secretion system (*ess* locus), staphyloxanthin biosynthesis (*crtM*, *crtN*, *crtP*), and quorum sensing (e.g. *agrA*) (**Figure 4**, **Table S5**). Individually, these pathways have been shown to bolster the ability of *S. aureus* to establish infection and evade the host immune system.^{52–54} Our results suggest that infection by Evo2 can lead MRSA to reduce the expression of all of these pathways, which could reduce the virulence of *S. aureus*.

Finally, we noted that both MW2 and LAC showed transcriptional changes that appear to be strain specific. For example, MW2 saw significant increase in the virulence factor *spa*, known to interfere with the host immune response and interface with other bacterial species. The presence of cell wall-bound Protein A has also been shown to decrease phage absorption, likely by masking WTA.⁵⁵ Further in MW2, we found that *tarM*, which adds α 1,4-GlcNAc to WTA, was strongly up-regulated ($\log_2$ ratio = 6.63). This is in line with previous findings showing that elevated *tarM* and α 1,4-GlcNAc-WTA can lead to phage resistance in MRSA. The LAC showed an increase in the expression ($\log_2$ fold change >3.8) of the hemolytic cytotoxin genes *lukD/E*, which lyses host cells and targets neutrophils.⁵⁶ We do not know whether the increased expression of these genes results in a greater level of protein production and secretion, but these transcriptional changes could represent a potential “trade-up” associated with phage resistance. Additional studies will be needed to fully assess the physiological and ecological effects of these upregulated genes in MRSA. Altogether, our RNA-seq results suggest that phage infection and resistance in MRSA cause significant transcriptional changes across a wide range virulence, metabolic, and cell-wall associated genes.

Phage-treated MRSA strains display reduced virulence phenotypes

S. aureus is a highly virulent pathogen, relying on a vast array of toxins and immune evasion proteins to promote infection.⁵⁷ MW2 and LAC strains as models of MRSA virulence.^{33,34} In light of our mutational and transcriptomic data, we hypothesized that phage-treated MRSA cells would display altered virulence phenotypes, in addition to reduced β -lactam resistance. We first tested the ability of MRSA strains that survived phage predation for their ability to form biofilms in a Crystal Violet assay. We found that Evo2 infection of MRSA252 resulted in a significant reduction in Crystal Violet absorption compared to the parental strain. However, we show no significant difference in Crystal Violet absorption between parental, mock- and phage-treated MW2 and LAC strains (**Figure S8**).

Next, we tested whether phage infection could affect the hemolysis of rabbit blood cells. Hemolysis is mediated by the secretion of toxins, notably alpha toxin encoded by the gene *hla*, and plays an important role in MRSA infection.⁵⁸ Expression of these toxins is regulated by virulence pathways that comprise numerous transcription factors, including *mgrA*, *arlR*, and *sarA*. Furthermore, in our RNA-seq results, we found that phage-resistant MRSA strains showed reduced expression of other cytotoxins. Parental MW2 and LAC colonies lysed rabbit blood cells on blood agar plates, producing distinct halos of clearance around the bacterial cells. For untreated LAC, the total area of hemolysis was on average 3-fold larger than that of untreated MW2 (~210 mm² vs ~70 mm², respectively); with MRSA252, by contrast, lysis was not detected (**Figure 5A**). Following treatment with Φ Staph1N, we observed that MW2 and LAC displayed a reduced area of hemolysis by 4 to 5-fold. With Evo2-treated cells, we found that in MW2 the fold reduction was comparable to that of Φ Staph1N-treated cells. However, for Evo2-treated LAC, loss of hemolysis was even more pronounced, with two of the replicates showing no detectable hemolysis. We note that neither MW2 nor LAC showed a reduction in transcript expression of *hla*. We posit that the loss of hemolysis could be driven by an inability of phage-evolved MRSA to secrete the toxin.

We next tested how phage infection affected cell agglutination (or clumping) in MRSA. *S. aureus* binds to fibrinogen, forming protective aggregates of bacterial cells. Clumping is thought to have several functions in the context of staphylococcal infections, facilitating adhesion to the host

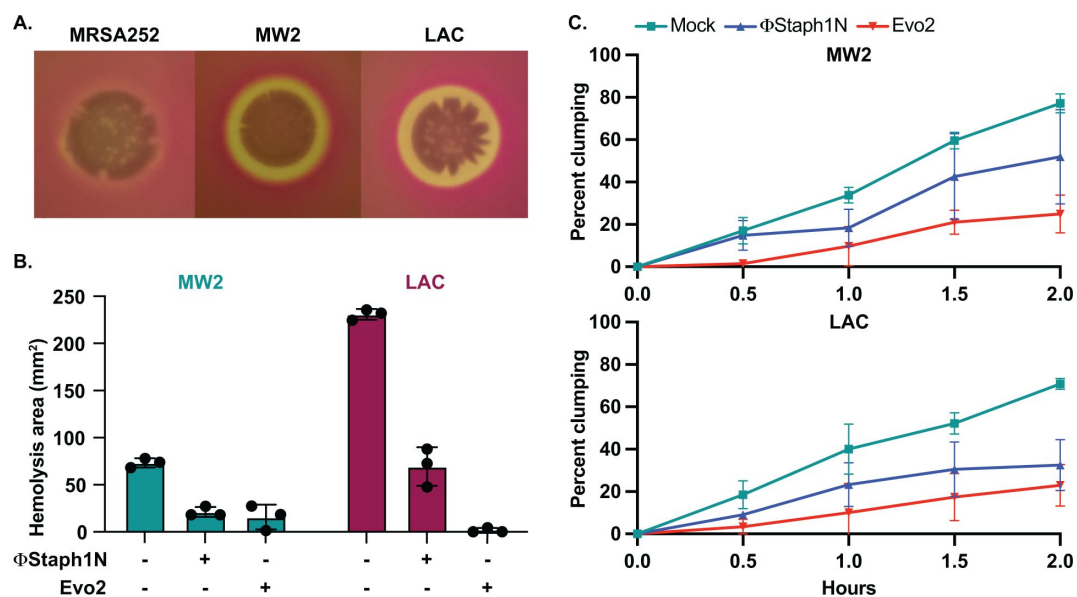


Figure 5.

Phage treatment of MRSA results in attenuated virulence phenotypes.

A. MW2 and LAC strains display hemolytic activity on rabbit blood agar plates, while MRSA252 does not. **B.** Phage-treated MW2 and LAC strains display reduced hemolysis compared to uninfected cells. **C.** Surviving cultures of MW2 and LAC treated with either ΦStaph1N (blue) or Evo2 (red) show reduced clumping rates compared to mock untreated cells (teal).

tissue. Clumps are also likely to be more resistant to clearance by the immune system, partly because they may be too large to be phagocytosed by neutrophils.³⁴ In our transcriptional data, we noted that several cell surface proteins known to reduce cell clumping, such as *ebh*, were over-expressed in phage-resistant MRSA. Horswill and colleagues found that de-repression of *ebh* reduces clumping. Indeed, phage-treated MW2 and LAC displayed less clumping than the mock-treated or parental strain. For MW2, Φ Staph1N infection resulted in a modest reduction, while Evo2 infection resulted in a reduction of approximately 3-fold (**Figure 5B**). In LAC, we found that both Φ Staph1N and Evo2 treatment resulted in comparable reductions of clumping in surviving cells. Overall, these phenotypic results align with our genetic and transcriptomic data, showing that phage infection can drive MRSA populations to reduced virulence phenotypes.

Combination treatment between Evo2 and β -lactam

The aforementioned results suggest that MRSA cells evolve phage resistance following infection, which is associated with trade-offs in virulence and β -lactam resistance. We next asked how MRSA populations would evolve under co-treatment with phage and β -lactam. In principle, these two simultaneous selective pressures could drive the evolution of resistance against both the phage and antibiotic, negating the trade-offs in drug resistance. To test this, we performed checkerboard assays with phage and oxacillin on MRSA252, MW2, and LAC. Serial dilutions of Evo2 or Φ Staph1N were mixed with serial dilutions of oxacillin on a 96-well plate (**Figure 6A**), after which MRSA strains were added to the plate and allowed to grow for 24 hours. Following 24 hours, one percent of the culture in each well was transferred into a fresh plate well with nonselective media, and the cultures were allowed to grow for another 24 hours (48 hours total). Throughout the experiment, the cell density was monitored by measuring the optical density in each plate well.

We first examined how MRSA grew in combinations of Evo2 and antibiotic (**Figure 6A**, top row). For MRSA252, cells grew at low levels of phage (MOI of 0.01 or less) and oxacillin (<0.125 $\mu\text{g/mL}$) (**Figure 6A**). LAC displayed greater sensitivity, displaying no detectable growth after 48 hours in the presence of phage, irrespective of the presence of oxacillin. For MW2, cells showed limited growth at MOIs <1 and oxacillin levels <0.125 $\mu\text{g/mL}$. For each strain, we picked cells from wells with an $\text{OD}_{600} >0.5$ and inoculated them into fresh, non-selective media. As a control, we also regrew cells that were treated with neither phage nor oxacillin (well B1). For MRSA252, two (E2 and F2) wells contained viable MRSA cells, forming turbid cultures; for MW2, six (D4, E2, E3, E5, F2, and F3) wells picked regrew (**Figure 6B**). We posit that cells in the failed cultures had reduced viability from the phage and antibiotic treatment. We tested these regrown cultures for their phage and oxacillin susceptibility. As expected, MRSA252 and MW2 cells from the B1 control wells were sensitive to Evo2, but resistant to oxacillin. In MRSA252, cells from E2 and F2 were resistant to Evo2 infection and exhibited a 1000-fold decrease in MIC against oxacillin. Similarly, in MW2, the six viable cultures exhibited Evo2 resistance and a 10- to 100-fold decrease in MIC. Altogether, these results match with those of MRSA that underwent single selection with Evo2.

We next analyzed how MRSA grew under Φ Staph1N/ β -lactam combinations (**Figure 6A**, bottom row). Neither MRSA252 nor LAC could grow in any Φ Staph1N/oxacillin combination; MW2, by contrast, grew across under a wide range of Φ Staph1N/oxacillin combinations. When Φ Staph1N-infected MW2 was treated with high (16 $\mu\text{g/mL}$) levels of oxacillin, recovered cells (wells D11 and E11) showed no reduction in MIC against oxacillin (**Figure 6C**). However, the plaquing efficiency of Φ Staph1N was reduced by 3 to 6 orders of magnitude. Whole genome sequencing revealed that these cells evolved a unique set of mutations different from those seen in the single phage treatment conditions (**Table S6**). However, when Φ Staph1N-infected MW2 was co-treated with low levels (<0.125 $\mu\text{g/mL}$) of oxacillin, recovered cells (C3) displayed strong phage resistance and a 100-fold reduction in oxacillin resistance, mirroring phenotypes observed in the single phage treatment experiments.

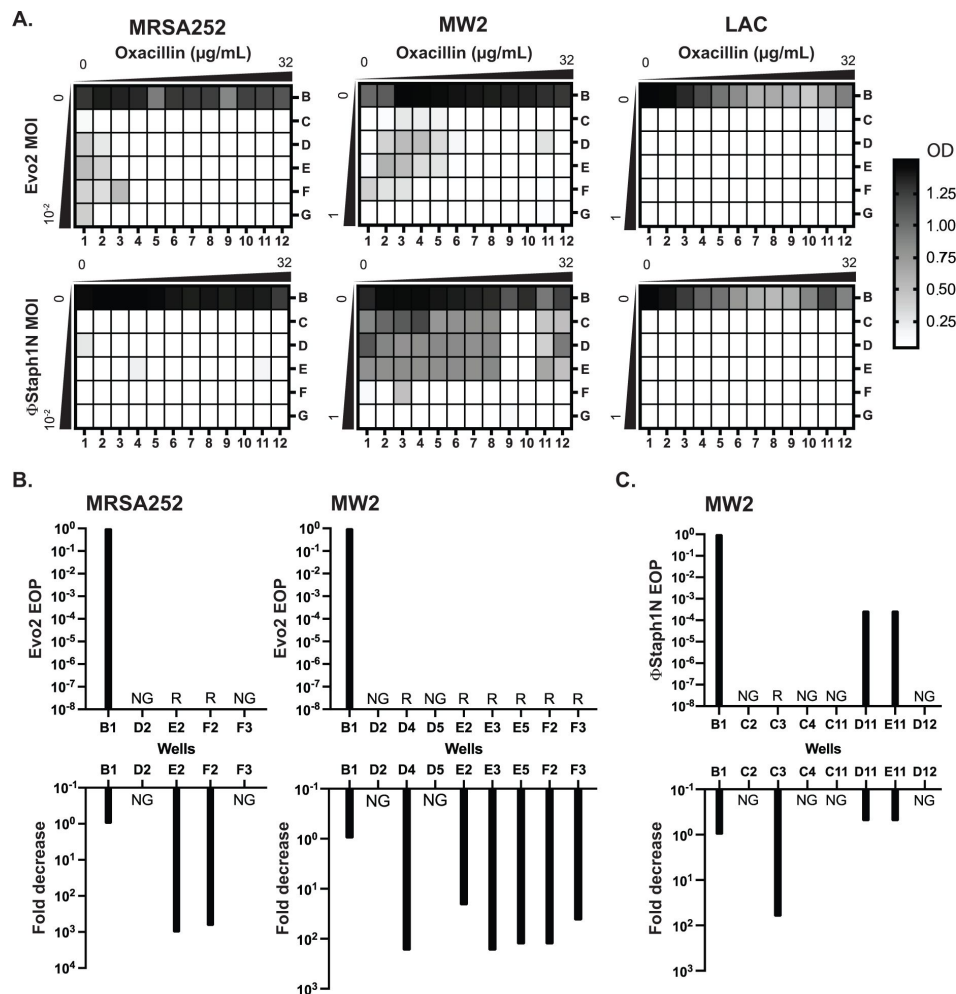


Figure 6.

Co-treatment of MRSA with bacteriophage and β -lactam.

A. Checkerboard assays of MRSA strains with gradients of oxacillin and Evo2 (top panels) or Φ Staph1N (bottom panels). The oxacillin gradient is a 2-fold serial dilution of drug concentration ($\mu\text{g/mL}$), while the phage MOI gradient is a 10-fold serial dilution of MOI. The rows and columns of each plate are labeled with letters and numbers, respectively. The black-white gradient in each well reflects the optical density of the culture and is the mean value from three biological replicates. MRSA strains co-treated with oxacillin and **(B)** Evo2 or **(C)** Φ Staph1N were tested for their phage resistance and oxacillin resistance. The letter/number combination reflects the well from which the cells were picked for analysis. Wells that could not produce a viable culture are labeled as NG (no growth). For wells that regrew, we calculated the efficiency of plaquing (EOP) of phage and measured the fold reduction in oxacillin MIC. Cultures that showed no detectable viral plaques are labeled as resistant (R).

We note that of the three MRSA strains tested, MW2 is the least sensitive to Φ Staph1N infection. We posit that the selective pressure exerted by high levels of β -lactam dominates over the selective pressure of Φ Staph1N, leading to the evolution of cells with continued β -lactam resistance and limited phage resistance. However, at lower β -lactam levels, the pressure exerted by phage infection predominates, leading to the rise of cells with complete phage resistance and trade-off in β -lactam resistance. This dose-dependent selection by oxacillin is not observed when a more active phage (e.g. Evo2) is used. While limited to one MRSA strain, these results suggest that different phage/ β -lactam combinations can produce divergent evolutionary outcomes in MRSA, each with potential clinical implications.

Discussion

Here we report the discovery that infection by certain phages can drive MRSA populations to evolve favorable genetic trade-offs between phage and β -lactam resistance. Exploiting genetic trade-offs has been proposed as a means to combat resistance in bacterial pathogens.¹⁸ Not only could phage treatments reduce the bacterial load of an infection, but also potentially resensitize bacterial populations to antibiotics against which they were previously resistant. We show that MRSA strains infected with *Kayviruses* Φ Staph1N, Evo2, and SATA8505 evolved resistance against phage, yet developed up to a 1000-fold loss in their MICs against β -lactam antibiotics. In addition, these evolved MRSA display reduced virulence phenotypes such as lower levels of hemolysis and clumping. Our findings show that phages can resensitize MRSA to β -lactams and even decrease their virulence, which are outcomes of significant biomedical value. However, not all staphylococcal phages can mediate these trade-offs: infection with Φ NM1y6, a phage of the *Dubovvirus* genus, did generate phage resistance in MRSA, but did not produce a drop in β -lactam resistance. A major future direction will be to determine which types of phages elicit these beneficial evolutionary trade-offs in MRSA.

Our results also paint a complex picture of MRSA evolution during phage infection. Whole genome sequencing revealed that MRSA strains evolved distinct mutation profiles following phage infection, suggesting a multitude of evolutionary paths that different bacterial strains can undertake to evolve resistance against phage. Not only did MRSA strains evolve distinct mutations, but individual, phage-resistant clones accumulated multiple mutations in their genome. Nonetheless, different MRSA displayed a convergence of phenotypes in the form of phage resistance, reduced β -lactam resistance, and attenuated virulence. We posit that this convergence is caused by the involvement of the cell wall in all three phenotypic outcomes. Phages must interface with the cell wall, β -lactams target proteins associated with cell wall maintenance, and many *S. aureus* virulence factors are embedded within the cell wall or are secreted through it. Thus, any modifications to the integrity or chemical composition of the cell wall by phage resistance will impact β -lactam sensitivity and virulence. Cell wall maintenance is controlled by numerous genes, ranging from single proteins involved in cell wall synthesis, such as *femA*, to transcriptional regulators, such as *mgrA*, that control the expression of cell wall synthesis genes. Thus, the mutational patterns observed in each MRSA strain could reflect genetic solutions that enable the bacteria to adapt to the phage predation, while also maximizing the fitness for that particular strain.

Strikingly, MRSA heavily modulated transcriptional profiles following phage infection. We believe these altered expression profiles are a consequence of the genomic mutations that emerged in the various transcriptional regulators. We observed that evolved cells downregulated genes involved in quorum sensing, type VII secretion, and a variety of toxins. It is intriguing to speculate how the down-regulation of these genes impacts MRSA interactions with other bacteria occupying the same ecological niche and with the host immune system. At the same time, MRSA strains also upregulated expression of select virulence factors, such as *spA*, which could represent “trade-ups.” Trade-ups are thought of as non-selected traits that are enhanced following selection (e.g. cross-

resistance between phage and antibiotic), which from a therapeutic perspective might be undesirable.⁵⁹ Future work will focus on assessing the risk of these trade-ups in light of the clinical benefit of reduced resistance and virulence.

Drug resistance in bacterial pathogens is an evolutionary problem and will require evolution-guided solutions to mitigate. Our findings highlight the ability of phages to dramatically alter the evolution and physiology of drug-resistant MRSA. Select phage treatments can force bacterial populations down evolutionary paths that make them vulnerable to antibiotics or the host immune system. Critically, this permits the re-deployment of agents that would otherwise remain ineffective, buying time for new drug discoveries. We therefore hope that our work may suggest avenues of research into new phage-based treatment strategies against MRSA and other drug resistance pathogens.

Online Methods

Strains and culture conditions

The bacterial strains used in this study are listed in **Table S1**. Unless otherwise indicated, all MRSA strains were grown in Brain Heart Infusion (BHI) media at 37 °C with shaking (235 RPM).

Plate-based plaque assay

Bacterial lawns were prepared by mixing 100 µL of an overnight culture with 5 mL of melted BHI agarose (top agar). The bacteria and top agar mixture were poured onto a solid BHI plate. The plate was dried for 10 minutes. 10-fold serial dilutions (10^0 – 10^{-7} unless otherwise noted) of phage were then spotted on the bacterial lawn. Plates were then incubated at 37 °C for 16 hours. Phage titer in plaque-forming units per µL (pfu/µL) was then calculated.

Phage infection assay

MRSA strains were plated onto BHI agar plates and grown overnight. Individual colonies from the parental strains (also referred to as P0) were picked. Single colonies were inoculated in a round bottle tube containing 5 mL BHI broth. The cultures were incubated at 37 °C, 235 RPM for 24 hours. The grown P0 cultures were then diluted 1:100 into fresh 5 mL BHI broth. At the early log phase (OD ~0.3), the bacterial cultures were treated with phage at an MOI of 0.1, unless indicated otherwise. The treated bacterial cultures were incubated at 37 °C with shaking (235 RPM) for 24 hours. The cultures were then passaged 1:100 into fresh 5 mL BHI broth. This passage was then grown at 37 °C, 235 RPM, for another 24 hours. Surviving cultures were then used for both phenotypic assays and sequencing experiments. As a negative control, MRSA strains were passaged using the steps described above without phage treatment (mock).

MIC assay

Bacterial lawns were normalized to contain 1×10^8 CFU/mL bacteria mixed with top agar for a total volume of 5 mL. The bacteria and top agar mixture is poured onto a solid BHI plate. The plate was dried for 10 minutes. MIC with increasing concentrations of antibiotics were placed on the semi-dried bacterial lawn and allowed to dry for 10 minutes. The plates are then incubated at 37 °C overnight. For analysis, the plates were imaged, and the MIC of the bacteria was determined. The MIC is determined at the edge of the inhibition ellipse intersects the side of the strip.

Rabbit blood hemolysis

Phage-treated or mock-treated cultures were diluted to an OD600 of 0.1, 5 µL of this dilution was spotted on rabbit blood TSB agar plates and incubated at 37 °C for 24 hours. The area of clearance was determined by the following formula: $[\pi (\text{diameter of clearance}/2)^2] - [\pi (\text{diameter of bacterial})^2]$

spot/2)²]

Clumping assay

Clumping assays were performed as described previously.⁶⁰ In short, overnight cultures were diluted 1:100 and incubated at 37 °C until the cultures reached an OD₆₀₀ of 1.5. At this point, 1.5 mL of culture was washed two times and resuspended with PBS. Lyophilized human plasma was added for a final concentration of 1.25%. Resuspended cells were left to sit statically at room temperature. 100uL were taken from the top of the cell suspensions in 30-minute intervals and the OD₆₀₀ was measured.

Biofilm assay

Biofilm assay was performed using the crystal violet method as outlined.⁶¹ In brief, overnight cultures grown in BHI at 37 °C were back diluted 1:100 into a 96-well bottomed microwell plate. The plates were incubated without shaking at 37 °C overnight. The contents in the plate were discarded and washed with PBS. Biofilm fixation was done with sodium acetate (2%). Crystal violet (0.1%) was used for staining followed by a final wash with PBS. Absorbance at 600 nm was read using a spectrophotometer.

DNA sequencing and genome assembly

Following published protocols, genomic DNA from bacteria and phage was isolated using phenol-chloroform extraction. Purified DNA was sent to Plasmidsaurus and SeqCenter for Nanopore and Illumina sequencing, respectively. Reference genomes for bacterial strains were assembled using Flye v2.9.3 with default settings for long-reads. This resulted in 1 singular contig assemblies for 252 (2902592bp, 125x) and MW2 (2820460, 600x), and 3 contigs for LAC (2907712, 645x). Phage assemblies for Evo2 and ΦStaph1N were done with the SPAdes assembler v3.15.5.

Open-reading frames (ORFs) were called on the assembled bacterial genomes using Prodigal v.2.6.3,⁶² resulting in a gene-feature file (GFF), and translated genes as .faa and .fna formats. We used BLASTp (Accessed June 4th, 2024), against the protein BLAST database swissprot_2023-06-28, with an expectation value cutoff of 0.001. The top hit for each ORF was used as the final functional annotation. Additionally, we annotate the genomes using Bakta v.1.10.3.

Mutation identification

A total of 27 genomic samples were collected. DNA was extracted and sent for long-read sequencing using Oxford Nanopore Technology (Plasmidsaurus, San Francisco, USA). Reads were filtered using filtlong v0.2.1 using default settings (<https://github.com/rrwick/Filtlong>) and with the -p flag 95 (keeping 95% of the best reads). Quality-filtered long reads were mapped against the respective genomes using minimap2 2.22-r1101,⁶³ resulting in 1 alignment file output per sample (.sam file). The read mapping software Minimap2⁶³ was selected because of its suitability to map long reads. Samtools v1.20⁶⁴ was used to convert the .sam files into .bam files, sort the bam file, index the bam files, and generate a coverage table for each position along the alignment.

Reference files (fasta and GFF files) and the 53 “sorted.bam” alignments files were imported into Geneious. Variant calling was performed using Geneious Prime Geneious Prime ® 2024.0.2 (www.geneious.com), using the custom settings: 10% coverage, minimum 95% variant frequency threshold, and the option for “Analyze effect of variants on translation” checked. Variant results and genome annotations table files were exported as a tab-separated-table, and visualized using R v4.4.0, mostly with the tidyverse package. Sequencing data processing, quality filtering, and mapping were performed at the Center for High-Throughput Computing (<https://chtc.cs.wisc.edu/>). BLASTp was performed on usegalaxy.eu (Accessed June 4th, 2024).

RNA purification

Parental and evolved MRSA strains were diluted 1:100 in BHI broth and incubated at 37 °C, 235 RPM until they reached an OD₆₀₀ of 1.5. 500 µL of culture were transferred into a microcentrifuge and 1 mL of RNAProtect Bacteria reagent (QIAGEN) was added. The mixture was vortexed for 5 seconds and incubated at room temperature for 5 minutes. The tubes were centrifuged for 10 minutes at 5000g, and the supernatant discarded. Bacterial pellets were resuspended in 80 µL of phosphate buffered saline and 10 µL of lysostaphin solution (1 mg/mL stock). The suspensions were incubated at 37 °C, with shaking, until the solution looked clear (~30 minutes). 10 µL of 10% sarkosyl was then added and the tube mixed, after which 300 µL of TRIzol reagent (Invitrogen) was added. RNA purification was performed following the protocol from the Direct-zol RNA Miniprep Plus kit (ZYMO Research). DNase I treatment was performed as recommended, and the samples were eluted in 75 µL of DNase/RNase-free water.

RNA sequencing and differential gene expression analysis

Purified RNA was prepared and sequenced on an Illumina sequencing platform at the UW-Madison Gene Expression Center. RNA-seq data was collected in the parental and evolved MRSA strains to assess differentially expressed genes. Cleaned RNA reads were mapped onto the LAC and MW2 reference genomes using bowtie2 v2.5.4,⁶⁵ and featureCount v2.0.8⁶⁶ from the software subreads was used to generate a read count matrix. Two read count matrices (one for LAC and one for MW2) were imported into R v.4.4.0 for processing with DESeq2 (version 1.44.0).⁶⁷ Figures were generated using the packages tidyverse (version 2.0.0) and EnhancedVolcanoPlots (version 1.22.0). To generate the volcano plots, we chose an adjusted p-value of 0.002 and a log₂ fold change (log₂FC) of < -2 or > 2. Multiple “unknown” genes were deemed significant (adjusted p-value < 0.002, abs(log₂FC) >= 2) in both LAC and MW2. To compare the results between the genomes, we performed a protein clustering analysis using MMseqs2 version b804fbc384e6f6c9fe96322ec0e92d48bccd0a42 between all the Bakta-generated amino acid sequences (.faa files) from LF and MW2.⁶⁸ Then we considered any protein sharing over 80% identity to be the “same” to generate a summary table showing up-regulation and down-regulation among the 2 genomes.

Checkerboard assay for phage-antibiotic synergy

The overnight cultures of MRSA252, Lac-Fitz, and MW2 were back-diluted 1:100 in BHI and incubated at 37 °C until the culture reached mid-log phase. The culture was then inoculated into each well of the 96-well plate containing a gradient of oxacillin and phage ([Staph1N or Evo2]). The oxacillin gradient was a 2-fold serial dilution, while the phage MOI gradient was a 10-fold serial dilution. The plates were then placed at 37 °C with shaking (235 RPM) for 24 hours.

Following 24 hours, each well from the plate was then passaged 1:100 into another 96-well plate with fresh BHI and grown at 37 °C with shaking (235 RPM) for another 24 hours. Following 48 hours of growth, the OD₆₀₀ was measured with a plate reader. Each checkerboard assay was performed in three biological replicates. A selection of surviving cells from individual wells (cells with an OD₆₀₀ reading of > .5) were then picked for phage plaquing and MIC assays. Efficiency of plaquing (EOP) was calculated by the following formula: plaque titer of treated cells/plaque titer of non-treated cells. Determine the mutation profile of survivor cells, genomic extraction, Illumina sequencing, and mutational analysis described above were used.

Supplemental data

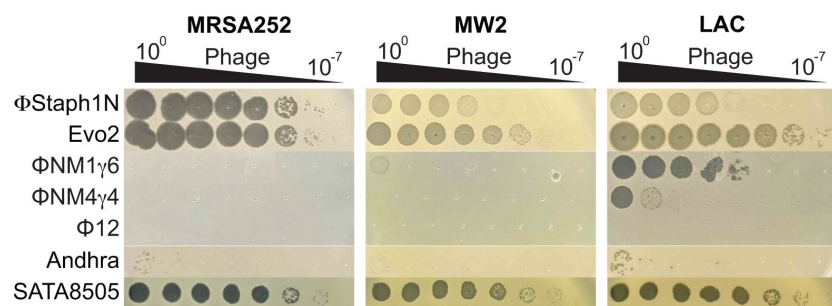


Figure S1.

Phage sensitivity of MRSA strains.

Efficiencies of plaquing of phages on MRSA252, MW2, and LAC. Phages were 10-fold serially diluted and spotted onto top agar overlays of each strain.

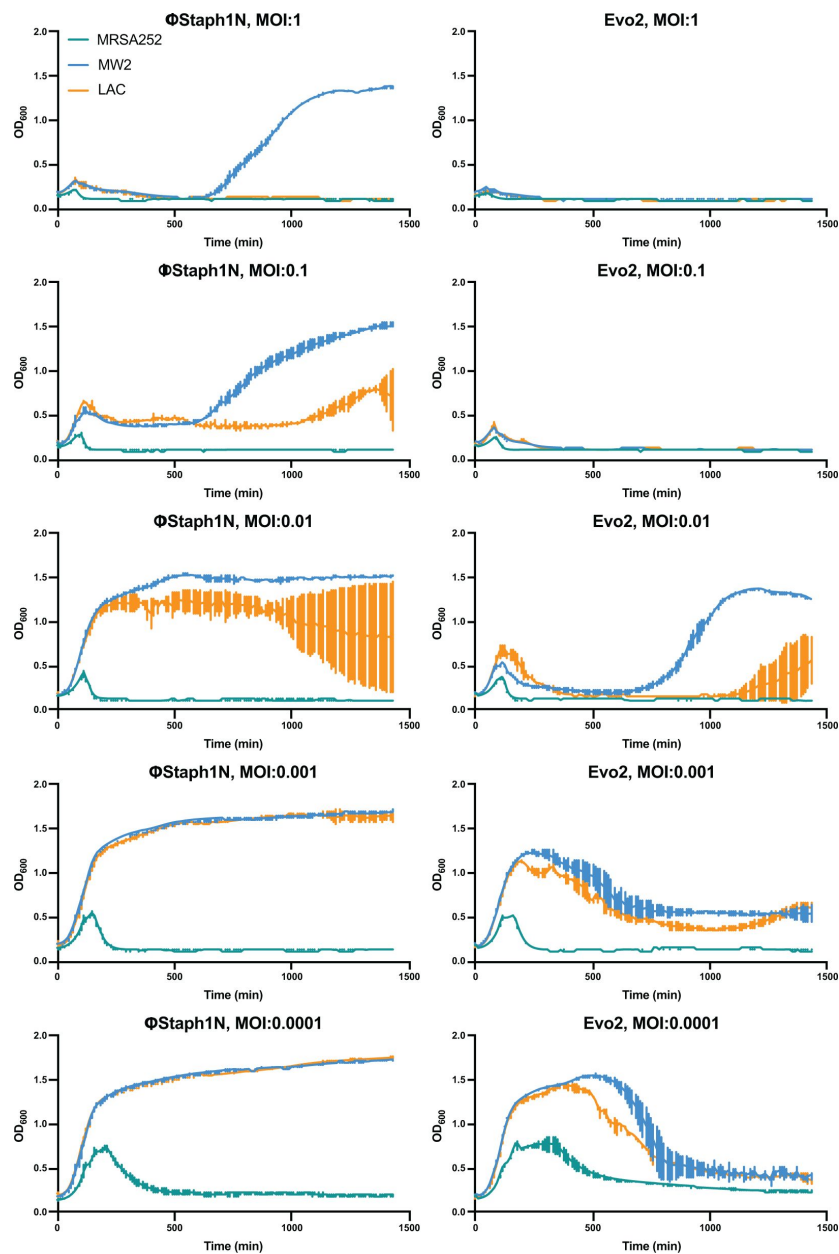


Figure S2.

Growth curves of MRSA strains under varying levels of phage infection.

MRSA252, MW2, and LAC cultures were infected with either Φ Staph1N or Evo2 at the indicated multiplicity of infection (MOI). The optical density (OD_{600}) of the cultures was monitored on an automated plate reader. Each condition was tested in 3 independent replicates.

Figure S3.

Isolation and sequencing analysis of Evo2.

A. Individual Evo2 plaques appeared in larger Φ Staph1N plaques on LAC. Individual plaques were isolated and propagated in liquid culture. Evo2 shows improved plaquing on MW2 and LAC. Plaquing data in the right panels are the same as in **Figure 2A**. **B.** Evo2 is a mutant form of Φ Staph1N with a nonsense mutation in ORF141. The A to C mutation (marked by the arrow) in Evo2 converts Serine 77 of ORF141 into a stop codon.

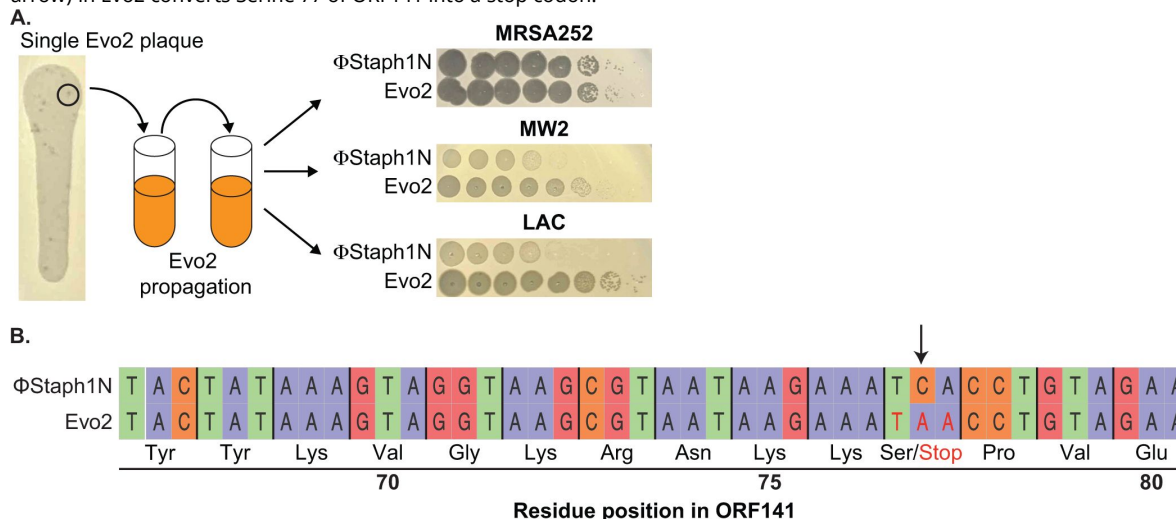


Figure S4.

Phage $\square$ NM1 γ 6 infection LAC does not drive the loss of β -lactam resistance.

A. LAC treated with $\square$ NM1 γ 6 evolves resistance against Φ NM1 γ 6, evidenced by the reduction of plaquing from the parental to the evolved populations. **B.** Evolved and parental LAC populations show comparable MICs against different β -lactams and vancomycin.

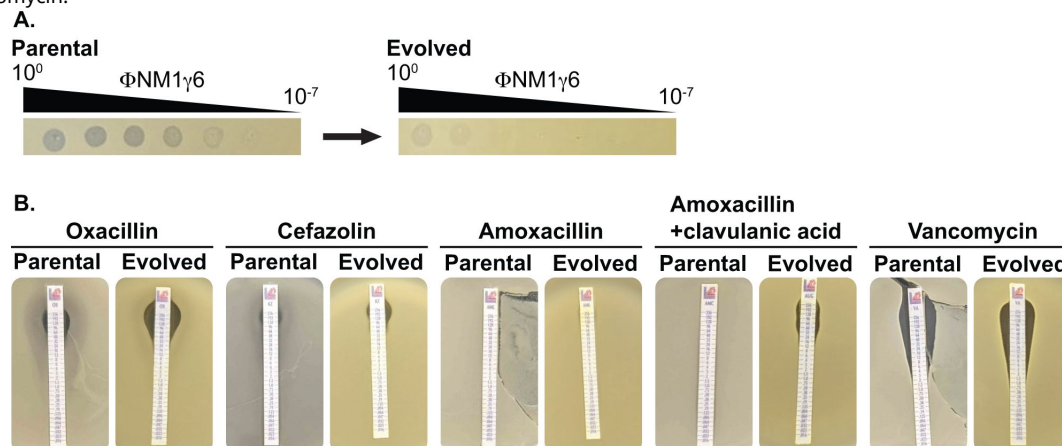


Figure S5.

Phage SATA8505 infection drives loss of oxacillin resistance.

A. MRSA strains MRSA252, MW2, and LAC treated with SATA8505 evolves resistance against SATA8505, evidenced by the reduction of plaquing from the parental to the evolved populations. **B.** Evolved and parental MRSA show reduced MICs against oxacillin.

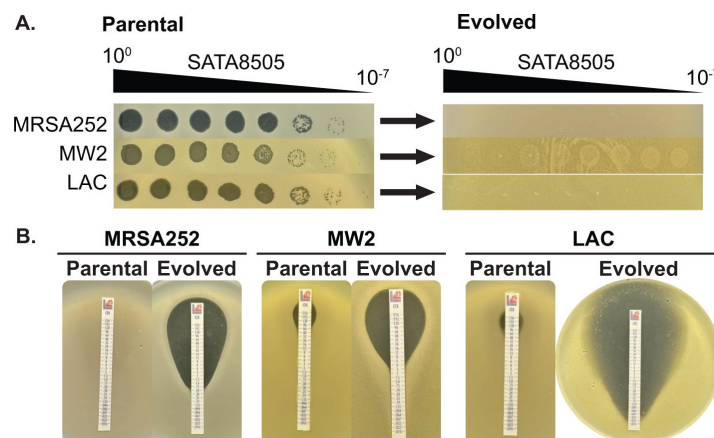


Figure S6.

Types of polymorphisms in MRSA strains following infection by phage or a mock treatment.

Plotted are the polymorphisms that were found in a gene with an assigned COG category.

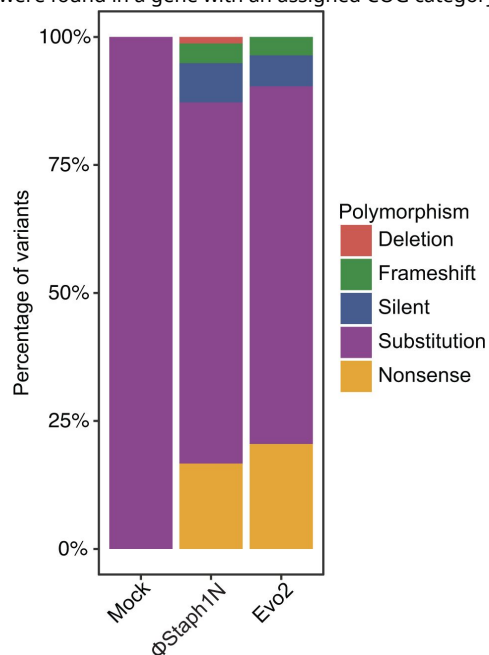


Figure S7.

Plaquing efficiency of Evo2 and Φ Staph1N on MW2 and LAC strains with knockouts in *mgrA*, *arl*, and *sarA*.

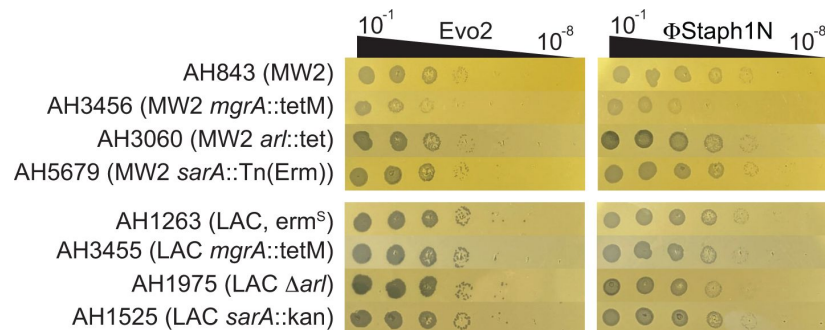
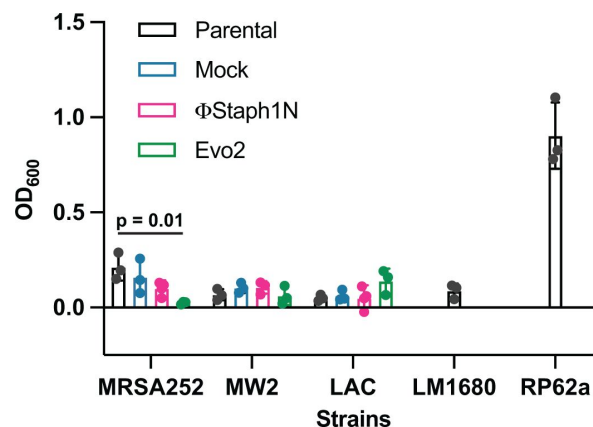


Figure S8.

Effect of phage infection on biofilm formation in MRSA strains.

Cultures were infected or mock-infected with either Φ Staph1N or Evo2. RP62a is a strain of *S. epidermidis* with known biofilm-forming capability, while LM1680 is a derivative of RP62a that has lost biofilm-forming ability.^{69,70} Biofilm biomass was assessed by staining with Crystal Violet. Solubilized crystal violet was quantified by measuring absorbance at 600 nm. Values represent averages and standard deviations of three replicates. Statistical significance was determined with a two-tailed t-test.



Strain Name	Comments	Source or Reference
MRSA MW2 (USA400)		Baba <i>et al.</i> , 2002
MRSA LAC (USA300)		Voyich <i>et al.</i> , 2005
MRSA252 (USA200)		Holden <i>et al.</i> , 2004
<i>S. epidermidis</i> RP62a	methicillin-resistant biofilm-producing <i>S. epidermidis</i>	Christensen <i>et al.</i> , 1982
<i>S. epidermidis</i> LAM1680	Derived from <i>S. epidermidis</i> RP62a; carries genomic deletion that inactivates biofilm production	Jiang <i>et al.</i> , 2016
ΦStaph1N		Łobocka <i>et al.</i> , 2012
Evo2	Derived from ΦStaph1N	This study
ΦNM1γ6	Lytic version of ΦNM1	Marraffini laboratory
ΦNM4γ4	Lytic version of ΦNM4	Marraffini laboratory
Φ12		Marraffini laboratory
Andhra	Infects <i>S. epidermidis</i>	Hatoum-Aslan laboratory
SATA8505	Isolated from the environment in this study	Environmental isolate; Pincus <i>et al.</i> , 2015
ADL1	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL2	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL3	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL4	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL5	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL6	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL7	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015

Table S1.

Bacterial strains and bacteriophages used in this study

ADL8	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL9	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL10	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL11	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL12	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL13	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL14	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL15	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL16	USA300, Patient isolate	Land <i>et al.</i> , 2015
ADL17	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL18	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL19	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL20	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL21	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL22	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL23	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL24	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL25	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL26	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL27	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL28	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL29	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015
ADL30	USA300, Patient isolate	Levin laboratory; Land <i>et al.</i> , 2015

Table S1. (continued)

Table S1. (continued)

AH1263	LAC, Erm ^S	Horswill 850 laboratory
AH3455	LAC <i>mgrA::tetM</i>	Horswill laboratory
AH1975	LAC Δ <i>arl</i>	Horswill laboratory
AH1525	LAC <i>sarA::kan</i>	Horswill laboratory
AH843	MW2	Horswill laboratory
AH3456	MW2 <i>mgrA::tetM</i>	Horswill laboratory
AH3060	MW2 <i>arl::tet</i>	Horswill laboratory
AH5679	MW2 <i>sarA::Tn(Erm)</i>	Horswill laboratory

Table S2.

MICs (μ g/mL) against oxacillin of MRSA strains treated with different MOIs of phage

	MRSA252					
	Φ Staph1N			Evo2		
MOI	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10 ⁻²	0.25	0.125	0.38	NG	2	0.5
10 ⁻³	NG	0.94	0.19	1	0.75	1
10 ⁻⁴	0.5	0.25	0.19	0.75	1	0.5
10 ⁻⁵	0.25	0.38	NG	0.38	NG	NG
Mock	>256	>256	>256	>256	>256	>256
	MW2					
	Φ Staph1N			Evo2		
MOI	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10 ⁻²	3	24	24	4	NG	NG
10 ⁻³	32	24	48	4	NG	NG
10 ⁻⁴	48	96	32	3	NG	NG
10 ⁻⁵	96	64	24	2	NG	NG
Mock	96	48	32	96	48	32
	LAC					
	Φ Staph1N			Evo2		
MOI	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10 ⁻²	NG	NG	2	0.064	NG	NG
10 ⁻³	NG	3	1.5	0.032	NG	NG
10 ⁻⁴	32	1.5	1	NG	NG	NG
10 ⁻⁵	32	16	0.38	NG	NG	NG
Mock	32	48	48	32	48	48

NG: no growth detected

		Mock			Evo2		
Strain	Antibiotic	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
MRSA252	Oxacillin	>256	>256	>256	0.38	0.75	0.5
	Rifampicin	0.047	0.032	0.012	0.023	0.047	0.023
	Mupirocin	0.75	0.5	1	0.5	0.5	0.38
	Erythromycin	>256	>256	>256	>256	>256	>256
	Teicoplanin	6	6	4	4	4	0.75
	Fosfomycin	12	8	8	8	8	6
	Daptomycin	2	3	2	2	2	2
MW2	Oxacillin	48	32	32	0.75	1	0.75
	Rifampicin	0.032	0.047	0.064	0.023	0.023	0.032
	Mupirocin	0.5	0.25	0.5	0.38	0.38	0.25
	Erythromycin	1	0.75	0.75	0.25	0.5	0.25
	Teicoplanin	2	1.5	1.5	1.5	1	1
	Fosfomycin	1	1.5	1.5	0.5	1	1
	Daptomycin	1.5	2	1.5	0.75	0.5	3
LAC	Oxacillin	48	24	64	0.19	0.064	0.047
	Rifampicin	0.047	0.047	0.047	0.032	0.032	0.047
	Mupirocin	0.5	0.75	0.75	0.5	0.5	0.5
	Erythromycin	3	3	3	1.5	1	2
	Teicoplanin	1	0.5	1	0.5	0.5	0.75
	Fosfomycin	6	6	12	1.5	12	1
	Daptomycin	0.5	3	1	0.064	2	0.75

Table S3.

MICs (µg/mL) of mock- or Evo2-treated MRSA strains against different antibiotics

Strain	ΦStaph1N	Evo2	ΦNM1γ6
RN4220	1.0E+00	1.0E+00	1.0E+00
ADL1	1.7E-02	2.7E+00	6.7E-01
ADL2	2.0E-01	3.3E+00	1.0E+00
ADL3	6.0E-02	2.0E+00	1.7E-02
ADL4	1.3E-03	1.0E+00	1.7E-01
ADL5	1.2E-02	2.7E+00	1.0E+00
ADL6	1.5E-02	1.7E+00	1.0E+00
ADL7	6.0E-01	1.7E+00	6.7E-01
ADL8	9.0E-02	2.0E+00	3.3E-01
ADL9	6.0E-03	1.3E+00	2.0E-04
ADL10	5.0E-01	1.3E+00	1.0E-03
ADL11	1.0E+00	1.0E+00	3.3E-01
ADL12	9.0E-04	2.0E+00	6.7E-07
ADL13	7.0E-02	2.7E+00	2.3E-01
ADL14	2.7E-01	3.3E+00	1.0E-05
ADL15	9.0E-02	3.0E+00	2.0E-02
ADL16	2.4E-01	5.6E+00	7.8E-02
ADL17	3.3E+00	1.0E+01	2.4E+00
ADL18	6.7E+00	1.4E+01	1.6E-02
ADL19	7.1E-01	1.6E+00	2.3E+00
ADL20	6.2E+00	7.8E+01	2.4E+00
ADL21	5.2E+00	5.6E+00	3.3E-06
ADL22	9.5E-01	2.0E+00	5.2E-01
ADL23	1.5E+00	3.3E+01	1.2E-01
ADL24	7.1E-01	5.6E+00	3.3E-06
ADL25	7.1E-01	1.8E+00	2.3E-01
ADL26	2.4E-02	3.9E+00	2.2E-03
ADL27	1.3E+00	7.8E+00	1.7E+00
ADL28	1.9E+00	8.9E+00	8.9E-02
ADL29	1.9E+00	1.0E+01	1.3E-04
ADL30	6.7E-01	1.4E+00	1.7E-05

*Phage EOPs on the clinical isolates are standardized to their respective EOP on the laboratory strain *S. aureus* RN4220.

Table S4.

Efficiencies of plaquing (EOPs)* of ΦStaph1N, Evo2, and ΦNM1γ6 on clinical isolates of USA300 (ADL1-30).

Gene	Strain	log ₂ Fold Change	p-value	p-value, adj.	Regulation
DBIAII_00174=PBCCJM_00186	MW2	-4.48	2.86E-07	1.47E-06	downregulated
DBIAII_00175=PBCCJM_00187	MW2	-3.80	1.41E-05	5.71E-05	downregulated
DBIAII_00176=PBCCJM_00188	MW2	-5.23	4.97E-27	1.14E-25	downregulated
DBIAII_00177=PBCCJM_00189	MW2	-5.38	1.09E-32	2.95E-31	downregulated
DBIAII_00178=PBCCJM_00190	MW2	-5.91	5.99E-12	5.14E-11	downregulated
DBIAII_00179=PBCCJM_00191	MW2	-6.25	1.96E-32	5.22E-31	downregulated
DBIAII_00183=PBCCJM_00195	MW2	-6.53	4.92E-14	4.88E-13	downregulated
DBIAII_00190=PBCCJM_00202	MW2	-5.14	5.18E-33	1.47E-31	downregulated
DBIAII_00256=PBCCJM_00269	MW2	2.47	5.12E-17	6.27E-16	upregulated
DBIAII_00461=PBCCJM_00477	MW2	2.44	1.38E-40	5.35E-39	upregulated
DBIAII_00467=PBCCJM_00482	MW2	8.48	1.72E-204	4.27E-202	upregulated
DBIAII_00594=PBCCJM_00608	MW2	2.76	5.73E-20	8.68E-19	upregulated
DBIAII_00595=PBCCJM_00609	MW2	2.43	3.40E-35	1.07E-33	upregulated
DBIAII_00603=PBCCJM_00617	MW2	2.40	1.38E-88	1.24E-86	upregulated
DBIAII_00607=PBCCJM_00621	MW2	-4.61	3.87E-04	1.23E-03	downregulated
DBIAII_00608=PBCCJM_00622	MW2	-3.92	1.24E-11	1.05E-10	downregulated
DBIAII_00998=PBCCJM_01016	MW2	-2.54	4.40E-86	3.65E-84	downregulated
DBIAII_00998=PBCCJM_01017	MW2	-2.54	4.40E-86	3.65E-84	downregulated
DBIAII_00999=PBCCJM_00948	MW2	-3.80	2.56E-79	2.05E-77	downregulated
DBIAII_00999=PBCCJM_01018	MW2	-3.80	2.56E-79	2.05E-77	downregulated
DBIAII_01132=PBCCJM_01149	MW2	-2.02	1.78E-06	8.29E-06	downregulated
DBIAII_01158=PBCCJM_01175	MW2	2.54	9.97E-26	2.07E-24	upregulated
DBIAII_01189=PBCCJM_01206	MW2	2.02	9.13E-37	3.05E-35	upregulated
DBIAII_01714=PBCCJM_01760	MW2	-2.60	1.99E-10	1.50E-09	downregulated
DBIAII_02017=PBCCJM_02076	MW2	-2.01	1.65E-18	2.19E-17	downregulated
DBIAII_02021=PBCCJM_02080	MW2	9.45	2.59E-257	1.16E-254	upregulated
DBIAII_02211=PBCCJM_02269	MW2	3.52	1.80E-71	1.26E-69	upregulated
DBIAII_02313=PBCCJM_02405	MW2	-2.61	2.39E-47	1.09E-45	downregulated
DBIAII_02313=PBCCJM_02408	MW2	-2.61	2.39E-47	1.09E-45	downregulated
DBIAII_02313=PBCCJM_02409	MW2	-2.61	2.39E-47	1.09E-45	downregulated
DBIAII_02340=PBCCJM_02433	MW2	-2.15	8.68E-33	2.40E-31	downregulated
DBIAII_02343=PBCCJM_02436	MW2	3.74	1.74E-123	2.79E-121	upregulated
DBIAII_02346=PBCCJM_02439	MW2	2.42	6.84E-24	1.32E-22	upregulated
DBIAII_02368=PBCCJM_02462	MW2	-4.74	1.59E-29	3.74E-28	downregulated
DBIAII_02391=PBCCJM_02484	MW2	2.20	4.47E-09	2.82E-08	upregulated

Table S5.

Genes with significant fold changes in MRSA MW2 and LAC strains following Evo2 infection.

DBIAII_02497=PBCCJM_02580	MW2	4.52	3.62E-133	6.24E-131	upregulated
DBIAII_02505=PBCCJM_02592	MW2	-2.39	2.13E-44	8.99E-43	downregulated
DBIAII_02650=PBCCJM_02740	MW2	-3.29	1.69E-73	1.22E-71	downregulated
DBIAII_02651=PBCCJM_02741	MW2	-3.11	3.35E-66	2.27E-64	downregulated
DBIAII_02652=PBCCJM_02742	MW2	-2.63	1.22E-52	5.95E-51	downregulated
ebh	MW2	7.13	0.00E+00	0.00E+00	upregulated
sarU	MW2	9.73	0.00E+00	0.00E+00	upregulated
lytN	MW2	7.47	9.68E-298	7.23E-295	upregulated
tarM	MW2	6.63	5.01E-292	2.80E-289	upregulated
fmhC	MW2	5.62	4.11E-223	1.53E-220	upregulated
efeO	MW2	4.03	2.34E-214	7.50E-212	upregulated
sarV	MW2	4.15	9.15E-214	2.56E-211	upregulated
sdrD	MW2	4.80	8.37E-185	1.88E-182	upregulated
efeB	MW2	4.24	5.85E-181	1.19E-178	upregulated
sarT	MW2	6.70	1.49E-179	2.79E-177	upregulated
yut	MW2	2.34	1.72E-113	2.58E-111	upregulated
spa	MW2	8.39	2.95E-112	4.13E-110	upregulated
fTR1	MW2	4.04	1.32E-104	1.74E-102	upregulated
ureF	MW2	3.81	1.83E-99	2.28E-97	upregulated
hysA	MW2	-2.89	4.28E-99	5.05E-97	downregulated
ureA	MW2	4.99	4.86E-97	5.44E-95	upregulated
gLY1	MW2	3.08	6.31E-96	6.73E-94	upregulated
ureG	MW2	3.34	1.33E-95	1.35E-93	upregulated
ureD	MW2	3.37	6.93E-92	6.75E-90	upregulated
capA	MW2	4.03	6.45E-91	6.02E-89	upregulated
wcaJ	MW2	4.05	1.56E-88	1.34E-86	upregulated
ureC	MW2	4.28	3.13E-78	2.42E-76	upregulated
fmtB	MW2	2.95	2.34E-75	1.75E-73	upregulated
argF	MW2	-2.01	6.12E-66	4.03E-64	downregulated
lpl9	MW2	-3.67	3.24E-65	2.08E-63	downregulated
sarS	MW2	3.20	4.10E-65	2.55E-63	upregulated
msaC	MW2	2.85	4.73E-64	2.87E-62	upregulated
ureE	MW2	3.79	4.19E-62	2.47E-60	upregulated
arcA	MW2	-2.86	4.87E-61	2.66E-59	downregulated
sdrE	MW2	-3.03	1.08E-59	5.79E-58	downregulated
ureB	MW2	4.73	2.10E-55	1.09E-53	upregulated
crtN	MW2	-2.84	4.66E-54	2.37E-52	downregulated
DBIAII_02627	MW2	2.28	7.23E-54	3.60E-52	upregulated
esaE	MW2	-2.67	1.37E-50	6.51E-49	downregulated

Table S5. (continued)

metN1	MW2	-2.11	1.05E-44	4.61E-43	downregulated
DBIAII_02296	MW2	-2.24	1.66E-44	7.15E-43	downregulated
cap8D	MW2	-3.71	2.74E-44	1.14E-42	downregulated
DBIAII_02618	MW2	-2.53	5.91E-44	2.41E-42	downregulated
tatC	MW2	2.29	7.15E-44	2.86E-42	upregulated
ywqE	MW2	3.54	1.46E-39	5.54E-38	upregulated
esaA	MW2	-2.65	1.16E-38	4.35E-37	downregulated
dprA	MW2	2.81	3.29E-38	1.21E-36	upregulated
sraP	MW2	2.25	1.12E-37	3.97E-36	upregulated
cwrA	MW2	4.32	1.34E-37	4.71E-36	upregulated
ldhD	MW2	4.09	3.07E-36	1.01E-34	upregulated
cap8E	MW2	-2.75	3.24E-36	1.05E-34	downregulated
psmA4	MW2	-5.07	4.23E-34	1.26E-32	downregulated
essA	MW2	-2.58	2.92E-32	7.61E-31	downregulated
feoA	MW2	4.26	3.43E-32	8.84E-31	upregulated
sasC	MW2	2.60	4.81E-32	1.23E-30	upregulated
arcD	MW2	-2.03	8.96E-31	2.21E-29	downregulated
cap8B	MW2	-3.46	8.89E-30	2.12E-28	downregulated
ceuD	MW2	2.22	1.85E-27	4.28E-26	upregulated
sceD	MW2	-2.28	5.16E-27	1.17E-25	downregulated
crtM	MW2	-2.82	8.48E-27	1.88E-25	downregulated
lytR	MW2	-2.24	3.47E-26	7.47E-25	downregulated
ydhF	MW2	-5.44	4.20E-26	8.88E-25	downregulated
lrgA	MW2	-3.86	6.84E-26	1.43E-24	downregulated
cap8G	MW2	-2.50	1.45E-24	2.93E-23	downregulated
tenA	MW2	2.33	2.86E-23	5.49E-22	upregulated
secY2	MW2	2.19	2.90E-23	5.51E-22	upregulated
ykoE	MW2	2.51	3.45E-23	6.44E-22	upregulated
hfq	MW2	2.22	9.87E-23	1.80E-21	upregulated
lip1	MW2	-2.76	1.28E-22	2.31E-21	downregulated
cap8C	MW2	-3.56	3.28E-22	5.79E-21	downregulated
ppnN	MW2	-5.45	4.85E-22	8.23E-21	downregulated
ssaA	MW2	-2.45	1.56E-21	2.58E-20	downregulated
thiE	MW2	2.03	3.60E-21	5.85E-20	upregulated
ftnA	MW2	-2.51	3.89E-21	6.27E-20	downregulated
pyrB	MW2	-2.12	4.39E-21	6.98E-20	downregulated
metL1	MW2	-3.62	4.72E-21	7.46E-20	downregulated
esxA	MW2	-2.14	9.07E-21	1.40E-19	downregulated
thiM	MW2	2.03	7.79E-20	1.16E-18	upregulated

Table S5. (continued)

essC	MW2	-2.02	1.14E-19	1.67E-18	downregulated
uraA	MW2	-2.40	5.59E-19	7.93E-18	downregulated
trpF	MW2	-2.59	8.66E-19	1.21E-17	downregulated
lytS	MW2	-2.41	1.25E-18	1.72E-17	downregulated
cntL	MW2	-2.14	1.39E-18	1.88E-17	downregulated
crtQ	MW2	-2.87	5.16E-18	6.65E-17	downregulated
essB	MW2	-2.02	9.68E-18	1.24E-16	downregulated
esxD	MW2	-2.58	1.70E-17	2.12E-16	downregulated
thiD	MW2	2.09	2.18E-17	2.70E-16	upregulated
mtlD	MW2	-2.08	5.25E-17	6.40E-16	downregulated
lrgB	MW2	-3.11	5.89E-17	7.13E-16	downregulated
argG	MW2	-3.33	6.39E-17	7.70E-16	downregulated
ceuC	MW2	2.51	1.12E-16	1.32E-15	upregulated
seh	MW2	-3.15	1.58E-16	1.83E-15	downregulated
asp1	MW2	2.13	2.27E-16	2.58E-15	upregulated
agrA	MW2	-3.53	3.32E-16	3.74E-15	downregulated
dapB	MW2	-2.40	1.97E-15	2.17E-14	downregulated
aur	MW2	-2.13	2.68E-15	2.93E-14	downregulated
ceuB	MW2	2.63	9.78E-15	1.03E-13	upregulated
trpC	MW2	-3.01	2.20E-14	2.27E-13	downregulated
pstS	MW2	2.82	2.94E-14	2.98E-13	upregulated
ehuA	MW2	-2.65	4.21E-14	4.21E-13	downregulated
agrB	MW2	-3.97	5.39E-14	5.33E-13	downregulated
cap8F	MW2	-2.28	9.21E-14	9.05E-13	downregulated
natK	MW2	-3.90	3.47E-13	3.26E-12	downregulated
yail	MW2	-5.84	2.07E-12	1.86E-11	downregulated
esxB	MW2	-2.30	4.70E-12	4.12E-11	downregulated
agrD	MW2	-3.17	1.57E-11	1.31E-10	downregulated
argH	MW2	-2.72	2.02E-11	1.67E-10	downregulated
lacA	MW2	2.03	2.87E-11	2.34E-10	upregulated
asd	MW2	-3.62	3.08E-11	2.51E-10	downregulated
cydD	MW2	-5.50	1.86E-10	1.40E-09	downregulated
dapA	MW2	-2.86	3.28E-10	2.37E-09	downregulated
nrdD	MW2	-2.34	3.30E-10	2.38E-09	downregulated
amaP	MW2	-2.55	3.99E-10	2.87E-09	downregulated
fetB	MW2	-2.37	6.18E-10	4.31E-09	downregulated
pstC	MW2	2.13	8.52E-10	5.84E-09	upregulated
uppP	MW2	-5.41	9.33E-10	6.38E-09	downregulated
vraX	MW2	2.08	3.19E-09	2.05E-08	upregulated

Table S5. (continued)

psmA3	MW2	-5.12	3.85E-09	2.46E-08	downregulated
cydC	MW2	-5.11	4.28E-09	2.72E-08	downregulated
hld	MW2	-6.48	6.58E-09	4.13E-08	downregulated
opp3C	MW2	-2.27	1.55E-08	9.39E-08	downregulated
psmA2	MW2	-7.28	1.71E-08	1.02E-07	downregulated
crtP	MW2	-2.04	4.01E-08	2.30E-07	downregulated
asp23	MW2	-2.11	1.61E-07	8.57E-07	downregulated
mgrA	MW2	-4.95	2.13E-06	9.90E-06	downregulated
DBIAII_00256=PBCCJM_00269	LAC	6.00	3.39E-43	9.59E-42	upregulated
DBIAII_00305=PBCCJM_00303	LAC	-3.16	5.34E-15	5.54E-14	downregulated
DBIAII_00401=PBCCJM_00416	LAC	2.50	5.60E-29	1.13E-27	upregulated
DBIAII_00461=PBCCJM_00477	LAC	4.54	0.00E+00	0.00E+00	upregulated
DBIAII_00594=PBCCJM_00608	LAC	3.83	1.69E-26	3.08E-25	upregulated
DBIAII_00595=PBCCJM_00609	LAC	3.20	3.32E-51	1.22E-49	upregulated
DBIAII_00646=PBCCJM_00662	LAC	-2.09	1.51E-53	5.94E-52	downregulated
DBIAII_00745=PBCCJM_00760	LAC	4.52	7.89E-44	2.34E-42	upregulated
DBIAII_00746=PBCCJM_00761	LAC	2.14	4.23E-34	1.00E-32	upregulated
DBIAII_00846=PBCCJM_01758	LAC	-2.18	6.03E-07	2.90E-06	downregulated
DBIAII_00931=PBCCJM_01018	LAC	-3.45	4.80E-115	5.29E-113	downregulated
DBIAII_00998=PBCCJM_01017	LAC	-3.33	1.32E-125	1.61E-123	downregulated
DBIAII_00999=PBCCJM_01018	LAC	-3.45	4.80E-115	5.29E-113	downregulated
DBIAII_01132=PBCCJM_01149	LAC	-2.84	3.00E-05	1.15E-04	downregulated
DBIAII_01291=PBCCJM_01317	LAC	2.21	4.45E-17	5.43E-16	upregulated
DBIAII_01322=PBCCJM_01351	LAC	2.04	2.20E-35	5.37E-34	upregulated
DBIAII_01476=PBCCJM_01506	LAC	-2.22	4.34E-15	4.55E-14	downregulated
DBIAII_01683=PBCCJM_01723	LAC	8.29	1.40E-187	2.49E-185	upregulated
DBIAII_01714=PBCCJM_01760	LAC	-4.62	1.36E-05	5.51E-05	downregulated
DBIAII_01820=PBCCJM_01874	LAC	3.93	2.68E-43	7.67E-42	upregulated
DBIAII_01982=PBCCJM_02038	LAC	-2.44	1.92E-12	1.56E-11	downregulated
DBIAII_01998=PBCCJM_02054	LAC	-2.33	5.45E-15	5.63E-14	downregulated
DBIAII_02017=PBCCJM_02076	LAC	-3.66	4.30E-18	5.69E-17	downregulated
DBIAII_02018=PBCCJM_02077	LAC	-3.20	1.28E-13	1.16E-12	downregulated
DBIAII_02019=PBCCJM_02078	LAC	-3.51	1.08E-14	1.08E-13	downregulated
DBIAII_02052=PBCCJM_02112	LAC	3.20	3.42E-44	1.03E-42	upregulated
DBIAII_02113=PBCCJM_02173	LAC	-3.50	4.30E-108	4.15E-106	downregulated
DBIAII_02114=PBCCJM_02174	LAC	-3.36	3.74E-111	3.77E-109	downregulated
DBIAII_02115=PBCCJM_02175	LAC	-3.51	3.56E-83	2.58E-81	downregulated
DBIAII_02169=PBCCJM_02227	LAC	2.86	8.92E-45	2.72E-43	upregulated
DBIAII_02211=PBCCJM_02269	LAC	4.04	3.27E-278	6.89E-276	upregulated

Table S5. (continued)

DBIAII_02222=PBCCJM_02281	LAC	-3.30	1.51E-12	1.24E-11	downregulated
DBIAII_02226=PBCCJM_02284	LAC	-2.02	3.66E-05	1.38E-04	downregulated
DBIAII_02278=PBCCJM_01758	LAC	-2.18	6.03E-07	2.90E-06	downregulated
DBIAII_02313=PBCCJM_02405	LAC	-2.43	1.01E-16	1.21E-15	downregulated
DBIAII_02315=PBCCJM_02405	LAC	-2.43	1.01E-16	1.21E-15	downregulated
DBIAII_02343=PBCCJM_02436	LAC	8.48	0.00E+00	0.00E+00	upregulated
DBIAII_02346=PBCCJM_02439	LAC	6.82	0.00E+00	0.00E+00	upregulated
DBIAII_02359=PBCCJM_02452	LAC	3.58	6.36E-23	1.03E-21	upregulated
DBIAII_02372=PBCCJM_02465	LAC	-2.60	9.73E-18	1.26E-16	downregulated
DBIAII_02502=PBCCJM_02587	LAC	-2.22	2.65E-80	1.76E-78	downregulated
DBIAII_02503=PBCCJM_02588	LAC	-2.44	5.23E-98	4.66E-96	downregulated
DBIAII_02504=PBCCJM_02589	LAC	-3.71	6.75E-19	9.31E-18	downregulated
DBIAII_02505=PBCCJM_02592	LAC	-3.59	2.99E-24	5.21E-23	downregulated
DBIAII_02650=PBCCJM_02740	LAC	-2.12	4.99E-51	1.81E-49	downregulated
DBIAII_02651=PBCCJM_02741	LAC	-2.06	3.29E-41	8.98E-40	downregulated
ebh	LAC	8.97	0.00E+00	0.00E+00	upregulated
sarV	LAC	6.03	0.00E+00	0.00E+00	upregulated
fmhA	LAC	4.10	0.00E+00	0.00E+00	upregulated
ywqE	LAC	7.62	0.00E+00	0.00E+00	upregulated
wcaJ	LAC	8.57	0.00E+00	0.00E+00	upregulated
rfbX	LAC	5.05	0.00E+00	0.00E+00	upregulated
capA	LAC	6.55	8.65E-305	2.00E-302	upregulated
sak	LAC	-4.41	1.98E-215	3.83E-213	downregulated
gLY1	LAC	5.45	3.62E-184	5.99E-182	upregulated
marR	LAC	2.23	3.49E-178	5.39E-176	upregulated
ldhD	LAC	4.54	7.20E-174	1.04E-171	upregulated
fmtB	LAC	7.62	4.46E-141	6.08E-139	upregulated
lytN	LAC	7.60	6.25E-128	8.04E-126	upregulated
phnD	LAC	6.78	9.07E-121	1.05E-118	upregulated
ureC	LAC	5.09	5.56E-113	5.85E-111	upregulated
phnC	LAC	6.31	2.22E-107	2.06E-105	upregulated
splD	LAC	5.29	1.21E-97	1.04E-95	upregulated
ureB	LAC	5.71	7.27E-89	6.01E-87	upregulated
hfq	LAC	3.04	1.15E-88	9.16E-87	upregulated
fmhC	LAC	6.00	3.49E-87	2.70E-85	upregulated
mrp	LAC	2.07	4.52E-85	3.38E-83	upregulated
phnE	LAC	5.12	1.82E-82	1.27E-80	upregulated
yut	LAC	3.93	1.39E-81	9.47E-80	upregulated
ureE	LAC	4.05	9.41E-79	5.89E-77	upregulated

Table S5. (continued)

ureA	LAC	6.43	5.77E-74	3.52E-72	upregulated
opp4A	LAC	8.69	1.05E-72	6.26E-71	upregulated
essB	LAC	-3.08	1.79E-66	1.04E-64	downregulated
msaC	LAC	3.76	2.60E-66	1.47E-64	upregulated
pyrC	LAC	-3.16	6.66E-66	3.68E-64	downregulated
splE	LAC	4.74	1.58E-65	8.51E-64	upregulated
esaA	LAC	-3.47	1.03E-64	5.33E-63	downregulated
splA	LAC	5.40	1.32E-63	6.50E-62	upregulated
ureF	LAC	4.22	7.55E-63	3.64E-61	upregulated
ureD	LAC	3.53	1.32E-61	6.19E-60	upregulated
splB	LAC	5.48	3.08E-56	1.35E-54	upregulated
ureG	LAC	3.22	3.15E-56	1.35E-54	upregulated
splC	LAC	5.10	5.34E-56	2.25E-54	upregulated
splF	LAC	4.32	6.76E-56	2.80E-54	upregulated
irrE	LAC	2.27	4.75E-53	1.83E-51	upregulated
essE	LAC	-3.11	1.70E-50	6.07E-49	downregulated
sarT	LAC	-2.75	6.41E-50	2.25E-48	downregulated
clfB	LAC	-3.75	1.93E-49	6.66E-48	downregulated
fnbA	LAC	-3.58	3.25E-48	1.11E-46	downregulated
lukE	LAC	4.13	1.11E-47	3.72E-46	upregulated
miaA	LAC	2.50	1.08E-46	3.58E-45	upregulated
PBCCJM_02590	LAC	-3.11	4.78E-46	1.56E-44	downregulated
brkB	LAC	-2.39	2.90E-45	9.34E-44	downregulated
lukH	LAC	-4.05	5.98E-45	1.87E-43	downregulated
isaB	LAC	2.70	7.03E-45	2.17E-43	upregulated
PBCCJM_02591	LAC	-2.40	1.08E-43	3.17E-42	downregulated
essA	LAC	-3.47	1.93E-43	5.59E-42	downregulated
esxC	LAC	-3.30	1.51E-42	4.22E-41	downregulated
pyrB	LAC	-3.26	5.06E-42	1.40E-40	downregulated
lukD	LAC	3.86	1.07E-40	2.88E-39	upregulated
argC	LAC	2.45	3.42E-40	9.11E-39	upregulated
lukG	LAC	-3.61	7.62E-39	2.01E-37	downregulated
esxA	LAC	-3.74	3.57E-38	9.30E-37	downregulated
lpl10	LAC	-2.20	1.49E-36	3.79E-35	downregulated
yzzA	LAC	-2.54	1.11E-34	2.67E-33	downregulated
tdcB	LAC	-4.27	8.20E-34	1.90E-32	downregulated
sasC	LAC	5.56	6.90E-33	1.58E-31	upregulated
pxpA	LAC	2.74	3.05E-31	6.79E-30	upregulated
norB	LAC	-4.11	1.53E-29	3.17E-28	downregulated

Table S5. (continued)

esxB	LAC	-3.00	4.26E-29	8.66E-28	downregulated
ugpQ1	LAC	-2.01	5.29E-28	1.01E-26	downregulated
essC	LAC	-3.24	9.15E-27	1.70E-25	downregulated
fetB	LAC	-2.71	8.96E-25	1.59E-23	downregulated
ridA	LAC	-2.04	2.46E-24	4.31E-23	downregulated
esxD	LAC	-2.60	1.69E-23	2.80E-22	downregulated
gtfB	LAC	-2.42	2.71E-23	4.42E-22	downregulated
gtfA	LAC	-2.64	7.59E-23	1.22E-21	downregulated
mntH	LAC	2.26	3.73E-22	5.73E-21	upregulated
carA	LAC	-2.37	4.97E-22	7.58E-21	downregulated
mtlD	LAC	-3.60	2.69E-21	4.02E-20	downregulated
dprA	LAC	3.02	4.72E-20	6.83E-19	upregulated
msrA	LAC	2.02	1.11E-19	1.59E-18	upregulated
lytS	LAC	-2.18	1.19E-19	1.70E-18	downregulated
PBCCJM_01714	LAC	-2.51	2.26E-19	3.21E-18	downregulated
mtlF	LAC	-3.52	2.45E-18	3.31E-17	downregulated
znuA	LAC	-2.03	1.03E-17	1.32E-16	downregulated
crtN	LAC	-2.20	1.24E-17	1.59E-16	downregulated
lacD	LAC	5.07	1.37E-17	1.74E-16	upregulated
lacC	LAC	4.91	1.40E-16	1.66E-15	upregulated
crtQ	LAC	-2.82	1.58E-16	1.87E-15	downregulated
cap8B	LAC	-2.78	1.81E-16	2.12E-15	downregulated
cwrA	LAC	2.60	2.74E-16	3.15E-15	upregulated
ICB5	LAC	-2.07	4.00E-16	4.52E-15	downregulated
lacB	LAC	4.99	1.29E-15	1.43E-14	upregulated
metL1	LAC	-2.45	1.32E-15	1.45E-14	downregulated
lacA	LAC	5.30	1.35E-15	1.48E-14	upregulated
lacF	LAC	5.10	5.02E-15	5.23E-14	upregulated
phnB	LAC	-2.35	1.01E-14	1.02E-13	downregulated
lrgB	LAC	-4.41	1.83E-14	1.80E-13	downregulated
lacE	LAC	4.38	2.52E-13	2.23E-12	upregulated
crtM	LAC	-2.60	2.52E-13	2.23E-12	downregulated
yocR	LAC	-2.31	4.87E-13	4.18E-12	downregulated
cap8C	LAC	-2.94	9.84E-13	8.20E-12	downregulated
InsB	LAC	-2.27	1.30E-12	1.08E-11	downregulated
wbil	LAC	-2.63	4.99E-12	3.95E-11	downregulated
lytR	LAC	-2.14	2.07E-11	1.55E-10	downregulated
fnbB	LAC	-2.02	4.53E-11	3.26E-10	downregulated
abgT	LAC	-2.48	6.66E-11	4.72E-10	downregulated

Table S5. (continued)

uraA	LAC	-2.34	1.20E-10	8.35E-10	downregulated
scn	LAC	-2.50	1.31E-10	9.08E-10	downregulated
opp4C	LAC	2.25	3.77E-10	2.51E-09	upregulated
merR	LAC	-2.33	1.10E-09	7.10E-09	downregulated
scb	LAC	-2.54	1.15E-09	7.39E-09	downregulated
efeB	LAC	3.49	2.28E-09	1.42E-08	upregulated
lacG	LAC	3.34	2.89E-09	1.77E-08	upregulated
efb	LAC	-2.49	4.59E-09	2.77E-08	downregulated
fTR1	LAC	3.32	1.01E-08	5.88E-08	upregulated
crtP	LAC	-2.61	1.97E-08	1.10E-07	downregulated
graF	LAC	-2.82	4.29E-08	2.32E-07	downregulated
ssaA2	LAC	-2.09	5.85E-08	3.12E-07	downregulated
crtO	LAC	-2.74	6.48E-08	3.41E-07	downregulated
yoze	LAC	-2.10	7.06E-08	3.71E-07	downregulated
amaP	LAC	-4.74	9.86E-08	5.10E-07	downregulated
lrgA	LAC	-4.81	1.92E-07	9.67E-07	downregulated
feoA	LAC	4.21	2.40E-07	1.21E-06	upregulated
esaB	LAC	-3.08	2.43E-07	1.22E-06	downregulated
pbp4	LAC	-2.57	3.05E-07	1.52E-06	downregulated
nikA	LAC	-2.24	3.78E-07	1.87E-06	downregulated
ycjR	LAC	-2.01	1.78E-06	8.03E-06	downregulated
sdrE	LAC	-2.12	2.54E-06	1.14E-05	downregulated
efeO	LAC	2.66	6.38E-06	2.73E-05	upregulated
agrA	LAC	-2.25	8.01E-06	3.39E-05	downregulated
opp3b	LAC	-2.13	1.05E-05	4.35E-05	downregulated
opp3C	LAC	-2.22	1.17E-05	4.79E-05	downregulated
sarS	LAC	-2.21	2.62E-05	1.02E-04	downregulated
fpr	LAC	-2.06	3.23E-04	1.03E-03	downregulated
asp23	LAC	-3.72	1.43E-03	3.98E-03	downregulated

DBIAII (MW2) or PBCCJM (LAC) prefix are the locus tag prefix as generated by Bakta (See Methods).

Table S5. (continued)

Gene	Description	Reference
rsaC ncRNA	modulate oxidative stress response and metal immunity	https://doi.org/10.1093/nar/gkz728
nrdF	class 1b ribonucleoside-diphosphate reductase subunit beta; beta subunit contains a metal-based cofactor; involved in DNA synthesis	10.1128/JB.183.24.7260-7272.2001 10.1074/jbc.M113.533554
fstAT ncRNA	Unknown	
rpoC	DNA-directed RNA polymerase subunit beta'	
tRNA	Transfer RNA	

Table S6.

Mutations in surviving MW2 cells co-treated with ΦStaph1N and oxacillin

Data and code availability

All the scripts can be found at: <https://github.com/patriciattran/mrsa-project>

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Additional information

Author contributions

MT and AJHV contributed equally to the work. MT, AJHV, and CYM designed the study; MT and AJHV performed all experiments; PQT performed the bioinformatic analysis; EDL and LT isolated and tested the bacteriophage SATA8505; CYM supervised the project. MT, AJHV, and CYM wrote the manuscript, with input from the other authors.

Additional files

Supplemental Table 1

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Reviewer #1 (Public review):

Summary:

These authors have asked how lytic phage predation impacts antibiotic resistance and virulence phenotypes in methicillin-resistant *Staphylococcus aureus* (MRSA). They report that staphylococcal phages cause MRSA strains to become sensitized to β -lactams and to display reduced virulence. Moreover, they identify mutations in a set of genes required for phage infection that may impact antibiotic resistance and virulence phenotypes.

Strengths:

Phage-mediated re-sensitization to antibiotics has been reported previously but the underlying mutational analyses have not been described. These studies suggest that phages and antibiotics may target similar pathways in bacteria.

Weaknesses:

One limitation is the lack of mechanistic investigations linking particular mutations to the phenotypes reported here. This limits the impact of the work.

Another limitation of this work is the use of lab strains and a single pair of phages. However, while incorporation of clinical isolates would increase the translational relevance of this work it is unlikely to change the conclusions.

Comments on revisions:

The authors have addressed my concerns.

<https://doi.org/10.7554/eLife.102743.2.sa2>

Reviewer #2 (Public review):

Summary:

The work presented in the manuscript by Tran et al deals with bacterial evolution in the presence of bacteriophage. Here, authors have taken three methicillin-resistant *S. aureus* strains that are also resistant to beta-lactams. Eventually, upon being exposed to phage, these strains develop beta-lactam sensitivity. Besides this, the strains also show other changes in their phenotype such as reduced binding to fibrinogen and hemolysis.

Strengths:

The experiments carried out are convincing to suggest such in vitro development of sensitivity to the antibiotics. Authors were also able to "evolve" phage in similar fashion thus showing enhanced virulence against the bacterium. In the end, authors carry out DNA sequencing of both evolved bacteria and phage and show mutations occurring in various genes. Overall, the experiments that have been carried out are convincing.

Weaknesses:

None. In the current version of the manuscript, I find the study complete.

<https://doi.org/10.7554/eLife.102743.2.sa1>

Author response:

The following is the authors' response to the original reviews:

We sincerely thank the reviewers for their thoughtful review and feedback. We believe that our work will provide valuable insights into how MRSA evolves under bacteriophage predation and stimulate efforts to use genetic trade-offs to combat drug resistance. We have substantially revised the paper and performed several additional experiments to address the reviewers' questions and concerns.

Summary:

(1) Testing for genetic trade-offs in additional *S. aureus* strains

We obtained 30 clinical isolates of the *S. aureus* USA300 strain that were isolated between 2008 and 2011 (see Table S1). We first tested the FStaph1N, Evo2, and FNM1g6 phages against this expanded strain panel and found that Evo2 showed strong activity against all 30 strains (Table S4). We tested whether Evo2 infection could elicit trade-offs in b-lactam resistance for a subset of these strains. We found that Evo2 infection caused a ~10-100-fold reduction in their MIC against oxacillin. This data is now incorporated into a revised Figure 2 in panel C.

(2) Testing additional staphylococcal phages

We isolated from the environment a phage called SATA8505. Similar to FStaph1N and Evo2, SATA8505 belongs to the *Kayvirus* genus and infects the MRSA strains MRSA252, MW2, and LAC. Phage-resistant MRSA recovered following SATA8505 infection also showed a strong reduction in oxacillin resistance (Figure S5). Furthermore, we confirmed that resistance against FNM1g6, which belongs to the *Dubowvirus* genes, does not elicit tradeoffs in b-lactam resistance (Figure S4). Sequencing analysis of FNM1g6 - resistant LAC strains showed a different mutation *fmcH*, which was not observed with the FStaph1N and Evo2 phages (Table 1). We have added this new data into the main text and supplemental figures and tables.

Future work will focus on obtaining comprehensive analysis of a wide range of phage families.

(3) Testing additional antibiotics

We also expanded our trade-off analysis include wider range of antibiotic classes (Table S3). Overall, the loss of resistance appears to be confined to b-lactams.

(4) Genetic analysis of ORF141

In order determine the function of ORF141, which is mutated in Evo2, we attempted to clone wild-type ORF141 into a staphylococcal plasmid and perform complementation assays with Evo2. Unfortunately, obtaining the plasmid-borne wild-type ORF141 has proven to be tricky, as all clones developed frameshift or deletions in the open reading frame. We posit that the gene product of ORF141 is toxic to the bacteria. We are currently working on placing the gene under more stringent expression conditions but feel that these efforts fall outside of the scope of this paper.

(5) Testing the effect of single mutants

Our genomic analysis showed that phage-resistant MRSA evolved multiple mutations following phage infection, making it difficult to determine the mechanism of each mutation alone. For example, phage-resistant MW2 and LAC evolved nonsense mutations in transcriptional regulators *mgrA*, *arlR*, and *sarA*. To test whether these mutations alone were sufficient to confer resistance, we obtained MRSA strains with single-gene knockouts of *mgrA*, *arlR*, and *sarA* and tested their ability to resist phage. We observed that deletion of *mgrA* in the MW2 resulted in a modest reduction in phage sensitivity (Figure S7). However, we did not observe any changes in the other mutant strains. These results suggest that phage resistance in these strains is likely caused by a combination of mutations. Determining the mechanisms of these mutations is the focus of our future work.

(6) Transcriptomics of phage-resistant MRSA strains

To further assess the effects of the phage resistance mutations, we performed bulk RNA-seq on phage-resistant MW2 and LAC strains and compared their differential expression levels to the respective wild-type strains. We picked these strains because our genomic data showed that they had evolved mutations in known transcriptional regulators (e.g. *mgrA*). Our analysis shows that both strains significantly modulate their gene expression (Figure 4). Notably, both strains upregulate the cell wall-associated protein *ebh*, while downregulating several genes involved in quorum sensing, virulence, and secretion. We have included this new data in Figure 4 and Table S5 and added an entire section in the manuscript discussing these results and their implications.

(7) Co-treatment of MRSA with phage and b-lactam

We performed checkerboard experiments on MRSA strains with phage and b-lactam gradients (Figure 6). We found that under most conditions, MRSA cells were only able to recover under low phage and b-lactam concentrations. Notably, these recovered cells were still phage resistant and b-lactam sensitive. However, under one condition where MW2 was treated with FStaph1N and b-lactam, we found that some recovered cells still had high levels of b-lactam resistance, showing a distinct mutational profile. We discuss these results in detail in the main text.

Reviewer # 1:

Strengths:

Phage-mediated re-sensitization to antibiotics has been reported previously but the underlying mutational analyses have not been described. These studies suggest that phages and antibiotics may target similar pathways in bacteria.

We thank Reviewer 1 for this assessment. We hope that the data provided in this work will help stimulate further inquiries into this area and help in the development of better phage-based therapies to combat MRSA.

Weaknesses:

One limitation is the lack of mechanistic investigations linking particular mutations to the phenotypes reported here. This limits the impact of the work.

We acknowledge the limitations of our initial analysis. We note (and cite) that separate studies have already linked mutations in *femA*, *mgrA*, *arlR*, and *sarA* with reduced b-lactam resistance and virulence phenotypes in MRSA, but not to phage resistance. For the other mutations, we could not find literature linking them to our observed phenotypes. We analyzed the effects of single gene knockouts of *mgrA*, *arlR*, and *sarA* on MRSA's phage resistance. However, as shown above, the results only showed modest effects on phage resistance in the MW2 strain (see Figure S7 and lines 309-317). We therefore believe that mutations in single genes are not sufficient to cause the trade-offs in phage/ b-lactam resistance. Because each MRSA strain evolved multiple mutations (e.g. MW2 evolved 6 or more mutations), we feel that determining the effects of all possible permutations of those mutations was beyond the scope of the paper.

However, to bridge the mutational data with our phenotypic observations, we performed RNAseq and compared the transcriptomes of un-treated and phage-treated MRSA strains (see Figure 4, Table S5, and lines 337-391). Our results show that phage-treated MRSA strains significantly modulate their transcript levels. Indeed, some of the changes in gene expression can explain for the phenotypic observations (e.g. overexpression of *ebh* can lead to reduced clumping). Further, the results shown some unexpected patterns, such as the downregulation of quorum sensing genes or genes involved in type VII secretion.

Another limitation of this work is the use of lab strains and a single pair of phages. However, while incorporation of clinical isolates would increase the translational relevance of this work it is unlikely to change the conclusions.

We thank the reviewer for this suggestion. We would like to clarify that MW2, MRSA252, and LAC are pathogenic clinical isolates that were isolated between 1997 and 2000's. However, we acknowledge that, because these 3 strains have been propagated for many generations, they might have acquired laboratory adaptations. We therefore obtained 30 USA300 clinical strains that were isolated in more recent years (~2008-2011) and tested our phages against them. We note that these clinical isolates (generously provided by Dr. Petra Levin's lab) were preserved with minimal passaging to reduce the effects of laboratory adaptation. We found that the Evo2 phage was able to elicit oxacillin trade-offs in those strains as well. (see Table S1, Table S7, Fig 2C, and lines 210 – 225)

For the phages, we had to work with phage(s) that could infect all three MRSA strains. That is why in our initial tests, we focused on Fstaph1N and Evo2, both members of the *Kayvirus* genus. Now in our revised work, we extend our analysis to FNM1g6, a member of the *Dubowvirus* genus, that also infects the LAC strain, but not MW2 and MRSA252. We find that FNM1g6 is unable to drive trade-offs in b-lactam resistance (see lines 229 – 238). Next, we analyzed the effects of SATA8505, also a member of the *Kayvirus* genus. Here, we observed that SATA8505 can elicit trade-offs in b-lactam resistance (see Figure S5 and lines 238 – 246).

These results suggest that not all staphylococcal phages can elicit these trade-offs and call for more comprehensive analyses of different types of phages.

Reviewer #1 (Recommendations for the authors):

Specific questions:

(1) The Evo2 isolate is an evolved version of phage Staph1N with more potent lytic activity. Is this reflected in more pronounced antibiotic sensitivity?

We did not observe that Evo2-treated MRSA cells showed more sensitivity towards b-lactams. However, we did observe that Evo2 was able to elicit these trade-offs at lower multiplicities of infection (MOI) (see lines 173 – 176 and Figure S2). Further, we did observe that Evo2 caused a greater trade-off in virulence phenotypes (hemolysis and cell agglutination) (see lines 416 - 419 lines 433 – 435, and Figure 5)

In our revisions, we also tested Evo2-treated MRSA against a wide range of antibiotics. We did not observe significant changes in MICs against those agents.

(2) Are there mutations in the SCCmec cassette or the Meca gene after selection against Φ Staph1N?

We did not observe any mutations in known resistance genes SCCmec or *blaZ*. Furthermore, we did not see any differential expression of those genes in our transcriptomic data (see lines 344 and 346).

(3) The authors report that phage Φ NM1y6 does not induce antibiotic sensitivity changes despite being effective against bacterial strain LAC. Were mutational sequencing studies performed with the resistant isolates that emerged against this strain? Can the authors hypothesize why these did not impact the virulence or resistance of LAC despite effective killing? How does this align with their models for Φ Staph1N?

We thank the reviewer for that insightful question. In our revised manuscript, we found that Φ NM1y6 elicits a point mutation in the *f_{mh}C* gene, which is involved in cell wall maintenance (see lines 326 – 335). To our knowledge, this point mutation has not been linked to phage resistance or drug sensitivity MRSA. Notably this mutation was not observed with Φ Staph1N or Evo2. We therefore speculate that Φ NM1y6 binds to a different receptor molecule on the MRSA cell wall.

(4) If I understand correctly, the authors attribute these effects of phage predation on antibiotic sensitivity and virulence to orthogonal selection pressures. A good test of this model would be to examine the mutations that emerge in antibiotic/phage co-treatment. This should be done.

We thank the reviewer for this suggestion. As described in the summary section above, we performed checkerboard experiments on MRSA strains with phage and b-lactam gradients (see lines 440 – 494 and Figure 6). We found that under most conditions, MRSA cells were only able to recover under low phage and b-lactam concentrations. Notably, these recovered cells were still phage resistant and b-lactam sensitive. However, under one condition where MW2 was treated with FStaph1N and b-lactam, we found that some recovered cells still had high levels of b-lactam resistance and only limited phage resistance, showing a distinct mutational profile (Figure S6). Under these conditions, we think that the selective pressure exerted by FStaph1N is “overcome” by the selective pressure of the high oxacillin concentration, a point that we discuss in the main text.

Reviewer #2 (Public review):

Summary:

The work presented in the manuscript by Tran et al deals with bacterial evolution in the presence of bacteriophage. Here, the authors have taken three methicillin-resistant S. aureus strains that are also resistant to beta-lactams. Eventually, upon being exposed to phage, these strains develop beta-lactam sensitivity. Besides this, the strains also show other changes in their phenotype such as reduced binding to fibrinogen and hemolysis.

Strengths:

The experiments carried out are convincing to suggest such in vitro development of sensitivity to the antibiotics. Authors were also able to "evolve" phage in a similar fashion thus showing enhanced virulence against the bacterium. In the end, authors carry out DNA sequencing of both evolved bacteria and phage and show mutations occurring in various genes. Overall, the experiments that have been carried out are convincing.

We thank Reviewer 2 for their positive comments.

Weaknesses:

Although more experiments are not needed, additional experiments could add more information. For example, the phage gene showing the HTH motif could be reintroduced in the bacterial genome and such a strain can then be assayed with wildtype phage infection to see enhanced virulence as suggested. At least one such experiment proves the discoveries regarding the identification of mutations and their outcome.

We thank the reviewer for this suggestion. We attempted to clone ORF141 into an expression plasmid and perform complementation experiments with Evo2 phage; however, all transformants that were isolated had premature stop-codons and frameshifts in the wild-type ORF141 insert that would disrupt protein function. We therefore think that the gene product of ORF141 might be toxic to the cells. We are currently working on placing the gene under more stringent transcriptional control but feel that these efforts fall outside of the scope of this paper.

Secondly, I also feel that authors looked for beta-lactam sensitivity and they found it. I am sure that if they look for rifampicin resistance in these strains, they will find that too. In this case, I cannot say that the evolution was directed to beta-lactam sensitivity; this is perhaps just one trait that was observed. This is the only weakness I find in the work. Nevertheless, I find the experiments convincing enough; more experiments only add value to the work.

We thank the reviewer for their comments. Because both phages and β -lactams interface with the bacterial cell wall, we posited that phage resistance would reduce resistance in cell wall targeting antibiotics. In our revisions, we have expanded our analysis to include a much wider range of antibiotic classes, including rifampicin, mupirocin, erythromycin, and other cell wall disruptors, such as daptomycin and teicoplanin. We did not observe any significant changes to the MICs of these other antibiotics (see Table S3 and lines 191-199). It therefore appears that the effects of these trade-offs are confined to beta-lactams.

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